

THE EFFECTS OF AQUI-S®20E (10% EUGENOL) SEDATION ON YELLOW PERCH *PERCA FLAVESCENS* AND TILAPIA *OREOCHROMIS NILOTICUS* FOR TRANSPORT

by

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A Thesis

Submitted in partial fulfillment of the requirements of the degree

MASTER OF SCIENCE

IN

NATURAL RESOURCES
(FISHERIES)

COLLEGE OF NATURAL RESOURCES

UNIVERSITY OF WISCONSIN

STEVENS POINT, WISCONSIN

June 27, 2013

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ABSTRACT

Fish transport costs are a substantial portion of the operational expenses of the aquaculture industry, especially as fuel costs continue to rise. Increasing fish loading density during transport could reduce expenses by enabling the transport of more fish mass per gallon of fuel. I hypothesized that the addition of AQUI-S®20E (10% eugenol, Lower Hutt, New Zealand), a eugenol based sedative, could allow increased loading densities without increasing mortality. In these studies, I used yellow perch *Perca flavescens* and Nile tilapia *Oreochromis niloticus* as model species to examine behavior, recovery from sedation, ammonia accumulation, eugenol uptake from water, survival and metabolic rates when exposed to AQUI-S®20E. AQUI-S®20E is a product currently being evaluated by the U.S. Food and Drug Administration Center for Veterinary Medicine as an immediate release finfish sedative. Fish were exposed to 0 to 300 mg L⁻¹ AQUI-S®20E (0 to 30 mg L⁻¹ eugenol) at loading densities up to three times the current industry standard during simulated transport and static respirometry. Concentrations of AQUI-S®20E ranging from 200 to 300 mg L⁻¹ (20 to 30 mg L⁻¹ eugenol) resulted in light sedation, >95% mean survival 7-day post-transport and decreased metabolic rates for yellow perch at loading densities up to 360 g L⁻¹ (three times the industry standard) in 17°C water. Tilapia held at 22°C showed minimal changes in metabolic rate and sedation when exposed to AQUI-S®20E concentrations up to 300 mg L⁻¹ (30 mg L⁻¹ eugenol), but had high mean survival (>90%) following a 10 h simulated transport at a loading density of 480 g L⁻¹ (two times the industry standard). Therefore, when using AQUI-S®20E to sedate fish during transport, it is important to consider how species and loading density will impact sedation. Loading density appears to have a negative effect on the level of sedation reached. Additionally, individual

species appear to respond differently to sedation. Overall, results indicated that AQUI-S®20E sedation during fish transport has the potential to allow fish haulers to transport at increased loading densities.

ACKNOWLEDGEMENTS

Completing this project has been a challenging and rewarding experience, which would not have been possible without many people. I would like to thank Dr. Chris Hartleb, my advisor and mentor, who taught me more about aquaculture and aquaponics than I expected. I would like to thank Dr. Ronald Crunkilton for taking an interest in my project and agreeing to serve on my committee. I am thankful for the opportunities to learn about aquaculture and career direction that Mark Gaikowski has given me through my employment at USGS. I am forever grateful to Dr. Kim Fredricks for the extensive time and thought she put into this project and for making long hours of research enjoyable. I would like to thank the U.S. Department of Agriculture – North Central Regional Aquaculture Center for providing the project funding. Special thanks to Jeff Meinertz, Scott Porcher, Justin Smerud and Sue Schleis for the long hours of data collection and comedic relief. I would like to thank Maggie Kenna and Steven Redman for taking care of the fish. Thanks to my friends Adam Plowman, Brian Rich, Dustin Drath and Joe Jakusz for shaping my college experience for the better. Thanks to Uncle Brad for encouraging me to pursue a graduate degree in fisheries. I am thankful for my mom, dad, and sisters who have always encouraged and supported me. Lastly, I especially want to thank Christa for motivating me to complete graduate school.

TABLE OF CONTENTS

TITLE PAGE	i
COMMITTEE SIGNATURES	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	v
LIST OF TABLES	viii
LIST OF FIGURES.....	x
LIST OF APPENDICES	xii
LITERATURE REVIEW.....	1
<i>Aquaculture Industry</i>	1
<i>Sedatives</i>	3
<i>Fish Transport</i>	5
<i>Thesis Objectives</i>	6
LITERATURE CITED.....	8
CHAPTER 1. SIMULATED TRANSPORT	18
ABSTRACT	18
INTRODUCTION.....	20
MATERIALS AND METHODS	23
<i>Study Animals</i>	23
<i>Pre-Eugenol Exposure</i>	23
<i>AQUI-S® 20E Calculation</i>	25
<i>Eugenol Exposure</i>	25
<i>Post-Eugenol Exposure</i>	27
<i>Statistical Analyses</i>	28
RESULTS.....	29
<i>Water Chemistry</i>	29
<i>Behavior and Eugenol Depletion – Yellow Perch</i>	29
<i>Ammonia – Yellow Perch</i>	30
<i>Recovery – Yellow Perch</i>	31
<i>Survival – Yellow Perch</i>	32
<i>Behavior and Eugenol Depletion – Tilapia</i>	32
<i>Ammonia – Tilapia</i>	33
<i>Recovery – Tilapia</i>	34
<i>Survival – Tilapia</i>	35
DISCUSSION	36
<i>Behavior During AQUI-S®20E Exposure</i>	37
<i>Eugenol Removal From Water</i>	40

<i>Survival 7-Days Post-AQUI-S®20E Exposure</i>	41
<i>Recovery Times Following AQUI-S® 20E Exposure</i>	42
<i>Ammonia Accumulation</i>	43
CONCLUSION	45
<i>Future Research</i>	46
LITERATURE CITED	48
CHAPTER 2. RESPIROMETRY	72
ABSTRACT	72
INTRODUCTION.....	74
MATERIALS AND METHODS	77
<i>Study Animals</i>	77
<i>Respirometry Design</i>	77
<i>Loading Density Calculations</i>	78
<i>AQUI-S®20E Calculation</i>	79
<i>Respirometry Trials</i>	79
<i>Metabolic Rate Calculation</i>	80
<i>Statistical Analyses</i>	81
RESULTS.....	82
<i>Yellow Perch Density</i>	82
<i>Tilapia Density</i>	83
<i>Yellow Perch – Two Fish</i>	84
<i>Tilapia – Two Fish</i>	84
DISCUSSION	85
<i>Yellow Perch</i>	85
<i>Tilapia</i>	87
CONCLUSION	90
<i>Future Research</i>	90
LITERATURE CITED	92

LIST OF TABLES

Table 1.1. Mean (\pm SEM) of total length (TL) and wet weight (WT) for yellow perch. Wet weights were measured prior to each replicate and growth was accounted for in loading density calculations.....	56
Table 1.2. Mean (\pm SEM) total length (TL) and wet weight (WT) for tilapia. Wet weights were measured before each replicate and growth was accounted for in loading density calculations.....	57
Table 1.3. Assignment of treatment buckets. In total, 108 buckets were tested. Treatment schedule encompassed all possible combinations of loading density, eugenol concentration, and transport duration for yellow perch.....	58
Table 1.4. Assignment of treatment buckets. In total, 108 buckets were tested. Treatment schedule encompassed all possible combinations of loading density, eugenol concentration, and transport duration for tilapia.....	59
Table 1.5. Mean (\pm SEM) water quality parameters for yellow perch during eugenol exposure and 7 d post-exposure observation. Water quality parameters were measured at 0, 1, 2, 4, 6, 8 and 10 h during static eugenol exposure-and once-daily for the 7 d flow-through post-exposure observation. Data were pooled for all treatment groups and replicate.....	60
Table 1.6. Mean (\pm SEM) water quality parameters for tilapia during eugenol exposure and 7 d post-exposure observation. Water quality parameters were measured at 0, 1, 2, 4, 6, 8 and 10 h during static eugenol exposure-and once-daily for the 7 d flow-through post-exposure observation. Data were pooled for all treatment groups and replicates.....	61
Table 1.7. Mean (\pm SEM) % survival for yellow perch 7 d following exposure to eugenol. Treatments with different letters are significantly different from each other ($p < 0.05$).....	62
Table 1.8. Summary of mean (\pm SEM) recovery times (min) for tilapia placed in flow-through fresh water immediately after transfer from static exposure to eugenol. Recovery from sedation was defined as upright, active swimming and the absence of any signs of sedation for 100% of fish within the tank. Zero minutes represents immediate recovery when placed in fresh water.....	63
Table 1.9. Mean (\pm SEM) survival (%), for tilapia 7 d following exposure to eugenol. No significant differences were detected.....	64

Table 2.1. Mean (\pm SD) water quality parameters, total length (cm) and wet weight (g) during respirometry experiments. Temperature was measured and recorded while fish were in the respirometry chambers. pH was measured from stock solutions. Yellow perch had only one pH measurement recorded therefore no standard deviation was reported. Weight was measured prior to each trial and accounted for in loading density calculations..... 98

Table 2.2. Loading densities (\pm SD) calculated from measured fish weights for yellow perch and tilapia..... 99

LIST OF FIGURES

Figure 1.1. Eugenol removal from simulated transport buckets when yellow perch were exposed to 10 (top), 20 (middle) or 30 (bottom) mg L ⁻¹ eugenol at 120, 240 and 360 g L ⁻¹ loading densities. Water samples were analyzed with HPLC (Agilent HPLC System Model: 1200 Series) over 10 h during eugenol exposure.	65
Figure 1.2. Summary of yellow perch behavior throughout 10 h of static exposure to 10 (top), 20 (middle) or 30 (bottom) mg L ⁻¹ eugenol. Loss of equilibrium was quantified as > 20% of the fish displaying this behavior. Light sedation was defined as a calculated ET80 of ≤ 20% showing loss of equilibrium and lacking a flight response to hand movement over tank and/or net avoidance. Normal was defined as an observable physical response to an external stimulus and net avoidance. Eugenol concentration (mg L ⁻¹) in tanks during behavioral transition was also reported above bars.....	66
Figure 1.3. Mean (±SEM) total ammonia nitrogen (mg L ⁻¹) for yellow perch exposed to 0, 10, 20 and 30 mg L ⁻¹ eugenol for 2 (top), 6 (middle) or 10 h (bottom) at 120, 240 and 360 g L ⁻¹ loading densities. Different uppercase letters show significant main effects between loading densities and different lowercase letters show significant main effects between eugenol concentrations within each loading density on TAN (p<0.05). No interactions were found.....	67
Figure 1.4. Summary of mean recovery times (min) for yellow perch placed in fresh water immediately following exposure to eugenol for 2 (top), 6 (middle) and 10 h (bottom). Recovery from sedation was defined as upright, active swimming and the absence of any signs of sedation for 100% of fish within the tank.....	68
Figure 1.5. Eugenol removal from simulated transport buckets when tilapia were exposed to 10 (top), 20 (middle) or 30 (bottom) mg L ⁻¹ eugenol at 240, 360 and 480 g L ⁻¹ loading densities. Water samples were analyzed with HPLC (Agilent HPLC System Model: 1200 Series) over 10 h during eugenol exposure.....	69
Figure 1.6. Summary of tilapia behavior throughout 10 h of static exposure to 10 (top), 20 (middle) or 30 (bottom) mg L ⁻¹ eugenol. Loss of equilibrium was quantified as > 20% of the fish displaying this behavior. Light sedation was defined as a calculated ET80 of ≤ 20% showing loss of equilibrium and lacking a flight response to hand movement over tank and/or net avoidance. Normal was defined as an observable physical response to an external stimulus and net avoidance. Eugenol concentration (mg L ⁻¹) in tanks at behavioral transitions was also reported above bars.....	70

Figure 1.7. Mean (\pm SEM) total ammonia nitrogen (mg L^{-1}) for tilapia exposed to 0, 10, 20 and 30 mg L^{-1} eugenol for 2 (top), 6 (middle) or 10 h (bottom) at 240, 360 and 480 g L^{-1} loading densities. Different uppercase letters show significant main effects between loading densities and different lowercase letters show significant main effects between eugenol concentrations within each loading density on TAN ($p < 0.05$). No significant interactions were found.....	71
Figure 2.1. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia (top) and yellow perch (bottom) under low, medium and high loading densities exposed to 0, 10, 20 and 30 mg L^{-1} eugenol. Treatments with different letters are significantly different from each other ($p < 0.05$).....	100
Figure 2.2. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia (top) and yellow perch (bottom) exposed to 0, 10, 20 and 30 mg L^{-1} eugenol at low, medium and high loading densities. Treatments with different letters are significantly different from each other ($p < 0.05$).....	101
Figure 2.3. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia and yellow perch at a loading density of two fish per trial exposed to 0, 10, 20 and 30 mg L^{-1} eugenol. No interspecies rate comparison was analyzed. Treatments with different letters are significantly different from each other ($p < 0.05$).....	102

LIST OF APPENDICES

Appendix A. Studies evaluating sedation with eugenol or clove oil..... 17

LITERATURE REVIEW

Aquaculture Industry

Worldwide, the aquaculture industry produced 55.7 million tonnes of fish, crustaceans and mollusks for an estimated \$105.3 billion in 2009 (FAO 2009). When combined with capture production, which produced nearly 90 million tonnes in 2009, more than 144.6 million tonnes of fish, crustaceans and mollusks were available for human use with about 84% directly for human consumption. While aquaculture produced approximately 38.5% of the world's seafood in 2009, production has steadily increased at a rate of 6.1% annually from 2001 to 2009 (FAO 2009). Specifically, freshwater aquaculture production increased 18% annually from 1978 to 2007 (National Bureau of Statistics 2008). In contrast, the worldwide capture fishery has plateaued at around 90 million tonnes annually from 1990 to 2009 (Diana 2009). With a growing worldwide demand for seafood, coupled with maximum harvest of capture fisheries, aquaculture will be responsible for fulfilling the increasing seafood demand in future years (Naylor et al. 2000; Bostock et al. 2010). Consequently, aquaculture is the fastest growing food production sector worldwide (Diana 2009).

Remarkably, China is responsible for 62.5% of the 55.7 million tonnes produced from aquaculture worldwide and total yield from Asia is nearly 90% of total aquaculture production (Bostock et al. 2010). Cyprinids, largely grass *Ctenopharyngodon idella* and silver carp *Hypophthalmichthys molitrix* were the primary species cultured for domestic human consumption in China (Broughton and Walker 2010) and were 40% of finfish culture worldwide (Diana 2009). However, the safety of food fish exported from China is a concern due to the unregulated use of pharmaceuticals, including some hazardous compounds that are applied

during the production process (Broughton and Walker 2010). While China is the frontrunner of exports worldwide, the United States and Japan receive more than 27% of total aquaculture imports (FAO 2009).

The United States had aquaculture sales of nearly \$1.1 billion from 4,309 registered farms in 2005, an increase of approximately 12% from \$978 million in 1998 (USDA 2006; FAO 2009) and equal to 1% of total world production. Aquaculture production in the U.S., though diverse, continues to be dominated by food fish production (USDA 2006). In 2005, aquaculture products sold were food fish (\$672 million), mollusks (\$203 million), crustacean (\$53 million), ornamental (\$51 million), baitfish (\$38 million) and sport fish (\$18 million). Food fish, about 60% of U.S. aquaculture, are strictly regulated by the U.S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition. Additionally, the FDA Center for Veterinary Medicine (CVM) regulates the use of antibiotics and pharmaceuticals and ensures the quality of food fish produced in the United States.

The FDA restricts the use of aquaculture drugs by requiring that all drugs undergo research to assess efficacy, target animal safety, human food safety, environmental impacts and to characterize the manufacturing process prior to approval (US FDA 2013). These stringent requirements are important for ensuring the quality and safety of aquaculture products in the United States. However, the strong regulations and lack of supportive research are also thought to put the U.S. at a competitive disadvantage, both in production capacity and costs, compared to countries where more drugs are available (Asche et al. 2008). Currently, only 16 drug trade names are approved by FDA, which are comprised of the following active ingredients formalin, hydrogen peroxide, oxytetracycline hydrochloride, oxytetracycline dihydrate, tricaine

methanesulfonate (MS-222), chorionic gonadotropin, florfenicol, and sulfadimethoxine/ormetoprim (US FDA 2013). Future research for evaluating drugs will be important for allowing the U.S. to compete with worldwide aquaculture markets.

Sedatives

Worldwide, benzocaine (Ross et al. 2007), clove oil (Cooke et al. 2004), AQUI-S® (Iverson and Eliassen 2009), lidocaine hydrochloride (Park et al. 2009), MS-222 (Pramod et al. 2010), quinaldine (Hasan and Bart 2007), 2-phenoxyethanol (Kaiser and Vine 1998), ketamine hydrochloride (Graham and Iwama 1990), metomidate (Crosby et al. 2010) and etomidate (Ross and Ross 2008) have been used to sedate fish. However, MS-222 is the only sedative approved in the U.S. for finfish sedation. MS-222 is an efficacious immobilizing and stress reducing anesthetic (Pramod et al. 2010; Gholipour et al. 2011). However, the FDA requires a 21-day withdrawal period for any uses of MS-222 on fish (US FDA 1997). Therefore, no human consumption of fish sedated with MS-222 may occur within 21-days post-sedation. For practical purposes, there is a strong need for an immediate-release sedative that can be used in aquaculture and by fisheries managers (Bowker and Trushenski 2012).

Recently, AQUI-S®20E (10% eugenol, Lower Hutt, New Zealand) has been studied for its sedation properties and to characterize its residues in fish after exposure. The active ingredient eugenol, 2-methoxy-4-prop-2-enyl-phenol, is the primary active ingredient in clove oil (70-90% by weight) and is derived from the plant *Eugenia caryophyllata* (Ross and Ross 2008). Raw clove oil also contains low concentrations of the active ingredients isoeugenol, acetyl eugenol and other turpenoid compounds. Clove oil is banned for use in food fish in the United States by the

FDA due to the potential carcinogenic properties of the other active compounds in clove oil. Eugenol is Generally Recognized as Safe (GRAS) by FDA for human use and is currently used in dentistry and in various herbal medicines (Curtis 1990; Fischer et al. 1990). Additional applications have shown eugenol as an antimycotic agent (Hussein et al. 2000), antiviral agent (Munding and Efferth 2008) and antibacterial agent (Rattanachaikunsopon and Phumkhachorn 2009).

Many fish have been evaluated for eugenol and clove oil sedation (Appendix A). Sedation is important for handling, tagging, enumerating, spawning and transporting of live fishes (Ross and Ross 2008). Results of various studies using eugenol or clove oil for general sedation in fishes have shown rapid induction and recovery times (Anderson et al. 1997; Gomes et al. 2011), low physiological disturbances (Cho and Heath 2000), rapid absorption and elimination from tissues (Kildea et al. 2004; Guenette et al. 2007) and the ability to minimize primary and secondary stress responses (Iverson et al. 2003; Deriggi et al. 2006; Palic et al. 2006).

However, few of these studies have evaluated products where eugenol is the only active ingredient. Moreover, even fewer studies have examined eugenol for light sedation. The few studies that have assessed eugenol or clove oil for light sedation of fish during transport have found lower net ion imbalances (Becker et al. 2012), reduced cardiovascular activity (Cooke et al. 2004), reduced stress response (Inoue et al. 2005; Hegyi et al. 2010) and reduced mortality (Iverson et al. 2009) relative to tanks containing no eugenol or clove oil. Additional research will be important to better understand the properties of eugenol sedation during fish transport.

Fish Transport

Fish are transported to markets as food fish or for stocking into the wild. Live transport can result in stressful conditions that can ultimately impact post-transport survival (Harmon 2009). Procedures taken during transport to minimize stress include: withholding feed 24 – 48 h prior to transport to minimize ammonia accumulation (Randall and Tsui 2002; Treasurer 2010), decreasing temperature prior to transport and maintaining proper temperature throughout transport (Golombieski et al. 2003; Pavlidis et al. 2003; Zhang et al. 2004), minimizing physical handling during tank loading and offloading (Cubero and Molinero 1997; Farrell et al. 2010), minimizing decreases in water quality (Erikson et al. 1997), adding salt to transport containers (Swanson et al. 1996) and proper loading densities (Urbinati et al. 2004; De Abreu et al. 2008; Carneiro et al. 2009; Pearson et al. 2009).

Loading densities directly limit the amount of fish that can be transported and changes as a function of temperature, duration of transport, life stage, and species (Pearson et al. 2009). Fish haulers often adhere to historical guidelines when choosing the loading density for transport (Piper et al. 1982). However, there is a lack of scientific literature to support the selection of these loading densities. Studies that evaluated survival post-transport found that for the highest loading densities tested, survival was high for golden shiner *Notemigonus crysoleucas*, matrinxá *Brycon amazonicus*, and silver catfish *Rhamdia quelen* (Golombieski et al. 2003; de Abreu et al. 2008; Carneiro et al. 2009; Pearson et al. 2009), which suggests that previously recommended loading densities were below the safe maximum densities. Since fish transport results in high costs and directly impacts potential profit (Pearson et al. 2009), fish

farmers and haulers would benefit economically by successfully transporting fish at higher loading densities than are currently recommended by the industry standard. Increasing loading densities would allow fish haulers to make fewer transport trips and minimize their transport expenses while increasing the mass of fish they haul. Further research assessing the maximum loading densities that allow high survival post-transport is needed.

Thesis Objectives

Research is needed to expand the drugs approved for aquaculture use in the United States. Currently, AQUI-S®20E (10% eugenol) is being evaluated under an Investigational New Animal Drug (INAD) for general sedation. To expand the utility of AQUI-S®20E under its current INAD and support future approval by FDA, research is needed to assess the effects of AQUI-S®20E for light sedation during fish transport. This thesis investigated the effects of AQUI-S®20E sedation on yellow perch *Perca flavescens* and Nile tilapia *Oreochromis niloticus* at loading densities up to three times the industry standard for simulated transport. Additionally, the effects of AQUI-S®20E on the metabolic rates of yellow perch and Nile tilapia at these higher loading densities was also assessed.

The specific objectives of this project were to:

1. Assess the use of AQUI-S®20E to enhance post-transport survival and increase loading density during transport.
2. Determine the optimal AQUI-S®20E concentration that would allow increased loading density during transport.

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Appendix A. Studies evaluating sedation with eugenol or clove oil.

Common Name	Latin Name	Sedative	Source
Silver catfish	<i>Rhamdia quelen</i>	Eugenol	Gomes et al. 2011
Zebrafish	<i>Danio rerio</i>	Clove oil	Sanchez-Vazquez et al. 2011
Senegalese sole	<i>Solea senegalensis</i>	Clove oil	Weber et al. 2011
Atlantic salmon	<i>Salmo salar</i>	Clove oil	Iverson et al. 2009
Rock bream	<i>Oplegnathus fasciatus</i>	Clove oil	Park et al. 2009
Hybrid striped bass	<i>Morone chrysops x M. saxatilis</i>	Clove oil	Sink and Neal 2009
Northern pike	<i>Esox lucius</i>	Clove oil	Zaikov et al. 2008
Rainbow trout	<i>Oncorhynchus mykiss</i>	Eugenol	Guenette et al. 2007
Nile tilapia	<i>Oreochromis niloticus</i>	Eugenol	Deriggi et al. 2006
Common carp	<i>Cyprinus carpio</i>	Clove oil	Hajek et al. 2006
Lake Victoria cichlid	<i>Haplochromis obliquidens</i>	Clove oil	Kaiser et al. 2006
Fathead minnow	<i>Pimephales promelas</i>	Eugenol	Palic et al. 2006
Matrinxa	<i>Brycon cephalus</i>	Clove oil	Inoue et al. 2005
Tambaqui	<i>Colossoma macropomum</i>	Eugenol	Roubach et al. 2005
European catfish	<i>Silurus glanis</i>	Clove oil	Velisek et al. 2005
Largemouth bass	<i>Micropterus salmoides</i>	Clove oil	Cooke et al. 2004
Tench	<i>Tinca tinca</i>	Clove oil	Hamackova et al. 2004
Silver perch	<i>Bidyanus bidyanus</i>	Clove oil	Kildea et al. 2004
Channel catfish	<i>Ictalurus punctatus</i>	Clove oil	Small 2003
Gilthead sea bream	<i>Sparus aurata</i>	Clove oil	Tort et al. 2002
Sockeye salmon	<i>Oncorhynchus nerka</i>	Clove oil	Woody et al. 2002
European perch	<i>Perca fluviatilis</i>	Clove oil	Hamackova et al. 2001
Red pacu	<i>Piaractus brachypomus</i>	Clove oil	Sladky et al. 2001
Chinook salmon	<i>Oncorhynchus tshawytscha</i>	Clove oil	Cho and Heath 2000
Walleye	<i>Sander vitreus</i>	Clove oil	Peake 1998
Rabbitfish	<i>Siganus lineatus</i>	Clove oil	Soto and Burhanuddin 1995

CHAPTER 1. SIMULATED TRANSPORT

ABSTRACT

Fish transport costs are a substantial portion of the operational expenses of the aquaculture industry, especially as fuel costs continue to rise. Increasing fish loading density during transport could reduce expenses by enabling the transport of more fish mass per gallon of fuel. I hypothesized that the addition of AQUI-S®20E (10% eugenol, Lower Hutt, New Zealand), a eugenol-based sedative, could allow increased loading densities without increasing mortality. In this study, I used yellow perch *Perca flavescens* and Nile tilapia *Oreochromis niloticus* as model species to examine behavior, recovery from sedation, ammonia accumulation, eugenol uptake from water and survival when exposed to AQUI-S®20E. AQUI-S®20E is currently being evaluated by the U.S. Food and Drug Administration Center for Veterinary Medicine as an immediate release finfish sedative. Fish were exposed to 0 to 300 mg L⁻¹ AQUI-S®20E (0 to 30 mg L⁻¹ eugenol) at loading densities up to three times the current industry standard during simulated transport. Concentrations of AQUI-S®20E ranging from 200 to 300 mg L⁻¹ (20 to 30 mg L⁻¹ eugenol) resulted in light sedation and >95% mean survival 7 days post-transport for yellow perch at loading densities up to 360 g L⁻¹ (three times the industry standard) in 17°C water. Tilapia held at 22°C only showed signs of sedation for less than 4 h when exposed to AQUI-S®20E concentrations up to 300 mg L⁻¹ (30 mg L⁻¹ eugenol), but had high mean survival (>90%) following a 10 h simulated transport at a loading density of 480 g L⁻¹ (two times the industry standard). Total ammonia nitrogen significantly increased as loading density increased, however, AQUI-S®20E was not effective reduce ammonia accumulation at any loading density. While sedation using AQUI-S®20E appears to be impacted by species and

loading density, results indicate that AQUI-S®20E has the potential to allow fish haulers to transport at increased loading densities.

INTRODUCTION

In aquaculture, fish are transported to markets as food fish or for stocking into the wild. Live transport can be stressful for fish and may ultimately impact post-transport survival (Harmon 2009). Procedures taken during transport to minimize the stress placed on fish include withholding feed 24 – 48 h prior to transport to minimize ammonia accumulation (Randall and Tsui 2002; Treasurer 2010), decreasing temperature prior to transport and maintaining proper temperature throughout transport (Golombieski et al. 2003; Pavlidis et al. 2003; Zhang et al. 2004), minimizing physical handling during tank loading and offloading (Cubero and Molinero 1997; Farrell et al. 2010), minimizing decreases in water quality (Erikson et al. 1997), adding salt to transport containers (Swanson et al. 1996), and proper loading densities (Urbinati et al. 2004; De Abreu et al. 2008; Carneiro et al. 2009; Pearson et al. 2009).

Loading densities directly limit the amount of fish that can be transported and changes as a function of temperature, duration of transport, life stage, and species (Pearson et al. 2009). Fish haulers often adhere to historical guidelines when choosing a loading density for transport (e.g. Piper et al. 1982), likely because there is a lack of scientific literature to support the selection of ideal loading densities for individual species. Studies that evaluated the application of increased loading densities on survival post-transport found that the highest loading densities tested resulted in high survival for golden shiner *Notemigonus crysoleucas*, matrinxa *Brycon amazonicus*, and silver catfish *Rhamdia quelen* (Golombieski et al. 2003; de Abreu et al. 2008; Carneiro et al. 2009; Pearson et al. 2009), which suggests that previously recommended loading densities were not being maximized. Recognizing that more research is needed to

evaluate elevated loading densities during transport, it was hypothesized that sedatives could be used to promote successful transport of increased loading densities.

Sedatives are important for handling, tagging, enumerating, spawning and transporting of live fishes (Ross and Ross 2008). While a suite of sedatives are available for use worldwide, only tricaine-methanesulfonate (MS-222) is approved for use in the United States. Use of MS-222, an efficacious immobilizing and stress reducing anesthetic (Pramod et al. 2010; Gholipour et al. 2011), is restricted by the 21 day withdrawal period assigned by the FDA (FDA 1997). Therefore, no human consumption of fish sedated with MS-222 may occur within 21 days post-sedation. For fish transport, there is a strong need for an immediate-release or short (<1 day) withdrawal period sedative that can be used in aquaculture and by fisheries managers (Bowker and Trushenski 2012). Specifically, AQUI-S® 20E (10% eugenol; Lower Hutt, New Zealand) has become a prime candidate as an immediate-release sedative for handling and light sedation (e.g. during transport).

Eugenol, 2-methoxy-4-prop-2-enyl-phenol, is the primary active ingredient in clove oil (70-90% by weight), derived from the plant *Eugenia caryophyllata* (Ross and Ross 2008) and often evaluated in the impure form of clove oil. Results of various studies using eugenol or clove oil for general sedation in fishes have shown rapid induction and recovery times (Anderson et al. 1997; Gomes et al. 2011), low physiological disturbances (Cho and Heath 2000), rapid absorption and elimination from tissues (Kildea et al. 2004; Guenette et al. 2007) and the ability to minimize primary and secondary stress responses (Iverson et al. 2003; Deriggi et al. 2006; Palic et al. 2006). Few studies have assessed eugenol or clove oil for light sedation directly for fish transport and found lower net ion imbalances (Becker et al. 2012), reduced

cardiovascular activity (Cooke et al. 2004), reduced stress response (Inoue et al. 2005; Hegyi et al. 2010), and reduced mortality (Iverson et al. 2009) relative to tanks containing no eugenol or clove oil. Recent studies by Meinertz et al. (2012, 2013) using the product AQUI-S®20E characterized the depletion of eugenol from rainbow trout tissues. Results of those studies indicated that eugenol quickly depleted from the tissues of rainbow trout at 17°C. Although various forms of eugenol have been evaluated for multiple applications of sedation, no previous studies have assessed the effectiveness of AQUI-S®20E to sedate fish at high loading densities.

Yellow perch *Perca flavescens* and Nile tilapia *Oreochromis niloticus* are two commercially and economically important fish in the United States (Malison 2000; El-Sayed 2006). Yellow perch are a cool water species cultured as both a food fish and for stocking (i.e. recreational harvest). Nile tilapia, a resilient warm water fish generating over \$3.7 billion in aquaculture production worldwide (FAO 2009), has drawn interest as a food fish in the United States. While yellow perch are sensitive to degrading water quality (Malison 2000), tilapia survive well in poor culture environments, which has promoted their increase in culture worldwide (Abdel Magid and Babiker 1975).

The objective of this study was to examine the effects of AQUI-S®20E sedation during the transport of yellow perch and Nile tilapia. Specifically, I was interested in identifying an AQUI-S®20E concentration that would allow yellow perch and tilapia haulers to safely increase loading densities beyond what is currently recommended.

MATERIALS AND METHODS

Study Animals

Yellow perch were hatched and reared in 17°C flow-through tanks at the Upper Midwest Environmental Science Center (UMESC), La Crosse, WI. Nile tilapia were obtained from Aquasafra Inc. (Bradenton, FL) as phenotypic males (>80% phenotypic males resulting from feed administered with 17-methyltestosterone as fry) then reared at UMESC in 24°C flow-through tanks. Yellow perch were used without regard to gender. Dissolved oxygen was measured during acclimation and grow-out phases. Yellow perch were fed Skretting extruded slow-sinking crumbles 1.6 mm salmon diet (Skretting USA, Tooele, UT) and tilapia were fed Purina Aquamax Fry Starter 200 1.2 mm and Purina Aquamax Grower 3/32 in. extruded pellet (Purina Mills, USA) to satiation once daily. Protocol approval from the UMESC Animal Care and Use Committee was gained prior to initiation of this study.

Pre-Eugenol Exposure

Fish were acclimated in a 700-L flow-through tank. Incoming water passed through a degassing column into a headbox before entering the tank containing fish. Three days before exposure, triplicate samples of fish were netted out of the source tank and group weighed (Sartorius balance Model BP 3100S) to obtain an average wet weight (g) per fish. The average weight was used to calculate the number of fish needed for each loading density. A typical sub-adult yellow perch loading density of 120 g L⁻¹ and sub-adult tilapia loading density of 240 g L⁻¹ was chosen based on surveys of fish haulers and a search of the literature. Fish haulers described these densities as the maximum safe loading densities for each species. To test the

effects of AQUI-S®20E as a sedative to increase loading densities beyond what is commonly used, yellow perch loading densities tested were 120 (100%), 240 (200%) and 360 g L⁻¹ (300%) and tilapia loading densities were 240 (100%), 360 (150%) and 480 g L⁻¹ (200%). Tilapia could not be increased to 300% of their typical loading densities, as was used for yellow perch, due to an unreasonable crowding of fish within tanks. Average wet weights were calculated for each of the three trials. The number of fish needed to achieve the desired loading densities was recalculated before stocking the observation tanks for each replicate to account for growth between eugenol exposures (Tables 1.1 & 1.2). Two days prior to eugenol exposure, fish were removed from the source tank and transferred to individual 8-L flow-through 18 x 32.5 x 22 cm clear, covered, plastic tanks. Fish were randomly chosen and distributed at predetermined loading densities. Blown air was supplied from a Sweetwater® pump (Model 8L94A) and diffused through airstones to all flow-through tanks and flow rates were set to a minimum of two tank exchanges per hour (265 mL min⁻¹). Water chemistry parameters were not measured prior to eugenol exposure. Fish were not fed 48 h prior to eugenol exposure, similar to what would occur before an actual transport.

AQUI-S®20E Calculation

The mass of AQUI-S®20E required to make stock solutions was calculated using the following equation (e.g. 10 mg L⁻¹ eugenol = 100 mg L⁻¹ AQUI-S®20E):

$$A = [(B * C) / 10^3] / 10\% \text{ active eugenol}$$

$$A = \text{AQUI-S®20E (g)}$$

$$B = \text{Volume of stock solution (L)}$$

$$C = \text{Target eugenol concentration (mg L}^{-1}\text{)}$$

Eugenol Exposure

AQUI-S®20E was weighed directly into 50-mL glass beakers (Sartorius Model 1712 MP8 and Sartorius Model LC 3201D balances). Four eugenol stock solutions were made by placing 20 L of source tank water into 50-L stainless steel tanks. AQUI-S®20E was then added from the 50-mL beakers directly into the stock tanks and mixed by hand for 1 min. Stock solutions contained 0, 10, 20 or 30 mg L⁻¹ eugenol, and concentrations were verified with a validated high performance liquid chromatography (HPLC) eugenol determination method (Agilent HPLC, Model: 1200 Series; Agilent Technologies, Santa Clara, CA). Briefly, 1.0 mL sample of the stock solution were removed with a BD™ 1.0-mL tuberculin (TB) syringe then filtered through a PALL Bulk Acrodisc® CR 13 mm, 0.2 µm pore filter (PALL Corporation, Port Washington, NY) into Agilent© 1.5 mL screw cap vials (Agilent Technologies, Santa Clara, CA). The sample collection location was at about the mid-depth of the stock solution. The following LC parameters were used: (a) mobile phase, water and methanol; (b) flow rate, 2.0 mL/min; (c) injection volume, 100 µL; (d) column temperature, 50°C; (e) guard cartridge, Phenomenex Security Guard™

(Torrance, CA); (f) analytical column, Phenomenex Synergi Max-RP, 4 μm , 80 \AA , 4.6 x 250 mm; (g) detector parameters: sample wavelength, 282 nm (maximum absorbance for eugenol with this LC system); sample bandwidth, 25 nm; reference wavelength, 360 nm; reference bandwidth, 80 nm; slit width, 16 nm; response time, 0.03 min; flow cell path length, 10 mm; and (h) run time, 4 min. Acceptable stock solution concentrations were within $\pm 10\%$ of target concentration. Samples were taken from the 0 mg L⁻¹ eugenol stock solution and analyzed to verify no eugenol contamination had occurred during mixing.

To simulate the static conditions of fish transport, thirty 3.8-L plastic buckets were filled to 0.75 L for yellow perch and 1.0 L for tilapia with stock solutions (0, 10, 20 or 30 mg L⁻¹ eugenol) and partially submerged in a water bath to maintain a temperature of 22 ± 1 °C for tilapia and 17 ± 1 °C for yellow perch. Trials were run in triplicate for each species (Tables 1.3 & 1.4). All buckets received compressed oxygen (Airgas UN1072) controlled by air regulators (Radnor Model: RC250-80-510 and AngelAqua[®] pressure gauges; Airgas, West Chicago, IL) throughout the entire 10 h eugenol exposure. Adjusting inflow of oxygen to the low volumes of water in the buckets during pilot studies resulted in large dissolved oxygen fluctuations. To eliminate poor dissolved oxygen as a potential variable resulting in mortality, compressed air was continuously supplied to all tanks and not adjusted while fish were in buckets. This resulted in saturation of dissolved oxygen throughout the static exposure. Prior to the addition of fish to buckets, a HACH[®] meter (Model: HQ40d; HACH Company, Loveland, CO) was used to measure pH (HACH[®] PHC101 probe; HACH Company, Loveland, CO), temperature, dissolved oxygen (HACH[®] LDO101 probe; HACH Company, Loveland, CO), and a 25 mL water sample was taken from each bucket to measure total ammonia-nitrogen (HACH[®] IntelliCAL Ammonia probe

ISENH3181; HACH Company, Loveland, CO). A 1.0 mL water sample was also taken from each bucket prior to the addition of fish to verify eugenol concentration at the start of the trial.

Fish were netted from the flow-through tanks and placed directly into the static buckets containing a 0, 10, 20 or 30 mg L⁻¹ eugenol solution. Water samples (1.0 mL) were taken from buckets at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 10 h and filtered as described above to determine eugenol depletion. Water chemistry (dissolved oxygen, pH and temperature) measurements were taken at 1, 2, 4, 6, 8 and 10 h in all buckets that contained fish. Fish behavior (response to hand movement, percent loss of equilibrium and net avoidance) were recorded at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 10 h from all buckets that contained fish. Fish were removed from buckets at 2, 6 and 10 h to simulate different transport durations. As described in Tables 1.3 and 1.4, following 2 h of eugenol exposure fish from nine of the buckets were netted and returned directly into the 8-L flow-through tanks. Recovery time (min) was measured from the time the fish entered fresh water to the time active, upright swimming returned. Nine buckets of fish were similarly placed in freshwater after 6 h and the remaining 12 buckets (9 eugenol buckets plus 3 control buckets) were individually placed in freshwater after 10 h. Total ammonia-nitrogen (mg L⁻¹) was measured in samples removed from the buckets once fish were transferred to fresh water.

Post-Eugenol Exposure

Fish were held in 8-L flow-through observation tanks for 7 days following eugenol exposure. All tanks were assessed daily for mortalities. Mortalities were removed and total length (TL; cm) and wet weights (g) were measured and recorded. Moribund fish were not

removed. Water chemistry consisted of pH, temperature and dissolved oxygen and was measured (HACH® meter Model: HQ40d) daily. Flow rates were measured and adjusted the first day to approximately two tank exchanges per hour (265 mL min^{-1}). Tanks were siphoned of waste and fed once daily.

On the seventh day, after removal of any mortalities and water quality measurements, fish were euthanized via an overdose (200 ppm) of MS-222 (Ross and Ross 2008). Total length (cm) and wet weight (g) were measured for 10 yellow perch and five tilapia from each tank. The remaining fish were counted, but morphometric measurements were not recorded.

Statistical Analyses

Data in tables and figures were reported as mean \pm standard error of the mean (SEM). The estimated time for 80% of fish to reach light sedation (ET80) was calculated for each treatment according to observed behavior. Homogeneity of variance was tested using Levene's median test. Normality of data was tested using the Shapiro-Wilk test. A log-transformation was used to normalize data when original data were not normally distributed. A one-way ANOVA and Tukey's HSD post hoc test were used to test for significant differences in survival for each loading density. In cases where transformations did not normalize the data, the non-parametric Kruskal-Wallis test or Mann Whitney U test with a Bonferroni adjustment was used to test significance. A two-way ANOVA and Tukey's HSD post hoc test were used to test for significant differences in total ammonia nitrogen (TAN) at 2, 6 and 10 h of simulated transport. Analyses were performed using R (R Core Team 2013; 64-bit, version 2.15.3) with the R Commander navigation interface (Fox 2005) and a minimum significance level of $P < 0.05$.

RESULTS

Water Chemistry

Water chemistry parameters for yellow perch and tilapia remained stable throughout simulated transport and during flow-through observation (Tables 1.5 & 1.6).

Behavior and Eugenol Depletion – Yellow Perch

Water samples (1.0 mL) were taken from the static buckets and analyzed for eugenol concentration and plotted over the 10 h simulated transport (Figure 1.1). All eugenol tanks, regardless of density, had eugenol concentrations less than 50% of the starting eugenol concentration by 2 h. However, eugenol depleted at different rates based on the loading density of fish contained within that bucket. Those buckets containing the highest loading density (360 g L⁻¹) had the fastest depletion of eugenol from the water. Those with the lowest density (120 g L⁻¹) had the slowest rate of eugenol depletion. Fish at a loading density of 240 g L⁻¹ removed eugenol from the buckets at an intermediate rate between the higher and lower densities. This trend of eugenol removal from simulated transport buckets was true for all eugenol concentrations.

Yellow perch behavior varied across treatment groups and loading densities (Figure 1.2). Because the percent of fish that lost equilibrium varied, the actual percent of fish with ventral or lateral sides upward within the bucket was recorded. If $\leq 20\%$ of the fish within the tanks exhibited loss of equilibrium and showed no response to hand movement and/or no net avoidance, they were assigned to the lightly sedated category. No yellow perch lost equilibrium

in the control or 10 mg L⁻¹ eugenol buckets. Two behavioral trends were observed at the 20 and 30 mg L⁻¹ eugenol treatments. First, as eugenol concentration increased, yellow perch exhibited prolonged loss of equilibrium. Second, within any given concentration, fish lost equilibrium for a shorter duration as loading density increased. Consequently, the estimated time for 80% of yellow perch to reach light sedation (ET80) was reduced at higher densities. Fish at the lightest density (120 g L⁻¹) and the highest eugenol concentration (30 mg L⁻¹) lost equilibrium for up to 8 h throughout the 10 h exposure. Light sedation, which is the desired level of sedation for fish transport, occurred for >7 h for yellow perch sedated with 30 mg L⁻¹ eugenol at 360 g L⁻¹ and 20 mg L⁻¹ at 240 g L⁻¹ loading densities. These were the longest durations of light sedation observed across all treatment groups. At all other eugenol concentrations and loading densities fish achieved light sedation for a maximum of 6 h. Fish in the control tanks (0 mg L⁻¹ eugenol) displayed no signs of sedation.

Ammonia – Yellow Perch

Total ammonia – nitrogen (TAN) was measured at 2, 6 and 10 h (Figure 1.3). There was a significant main effect of loading density on the amount of TAN in the buckets at 2 h ($F_{2,24} = 51.85$, $p < 0.01$). The Tukey's HSD post hoc revealed that the TAN accumulated in buckets was significantly lower at 120 g L⁻¹ than both 240 and 360 g L⁻¹ loading densities (both p 's < 0.01). The TAN for the 240 g L⁻¹ density was also found to be significantly lower than the 360 g L⁻¹ density ($p < 0.05$). Additionally, there was a main effect of eugenol concentration on the amount of TAN in the buckets at 2 h ($F_{3,24} = 3.515$, $p < 0.05$). Tukey's HSD post hoc showed that TAN in buckets was higher for 10 mg L⁻¹ eugenol relative to the 0 mg L⁻¹ control ($p < 0.05$). The

TAN accumulated in buckets was not significantly different between any other eugenol concentrations. There was a non-significant interaction effect between loading density and eugenol concentration ($F_{6,24} = 1.434$, $p > 0.05$).

There was a significant main effect of loading density on TAN levels at 6 h ($F_{2,22} = 62.745$, $p < 0.01$). Tukey's HSD post hoc showed that 120 g L⁻¹ had significantly lower TAN than 240 and 360 g L⁻¹ loading densities (both p 's < 0.01). The TAN at 240 g L⁻¹ density was also found to be significantly lower than TAN at 360 g L⁻¹ loading density ($p < 0.01$). There was a non-significant main effect of eugenol concentration ($F_{3,22} = 0.946$, $p > 0.05$) and a non-significant interaction effect of loading density and eugenol concentration on TAN ($F_{6,22} = 1.685$, $p > 0.05$).

A significant main effect of loading density on TAN levels at 10 h was found ($F_{2,22} = 20.840$, $p < 0.01$). Tukey's HSD post hoc showed significantly lower TAN for 120 g L⁻¹ relative to 240 and 360 g L⁻¹ densities (both p 's < 0.01), but no significant difference was found between 240 and 360 g L⁻¹ densities ($p > 0.05$). There was a non-significant main effect of eugenol concentration ($F_{3,22} = 1.146$, $p > 0.05$) and a non-significant interaction of loading density and eugenol concentration on TAN accumulation ($F_{6,22} = 0.142$, $p > 0.05$).

Recovery – Yellow Perch

Recovery from sedation (min) was visually observed immediately after transferring fish to fresh water (Figure 1.4). Control fish immediately recovered when placed in fresh water. Yellow perch exposed to 10 mg L⁻¹ eugenol recovered in less than 1.0±1.0 min, and most immediately recovered. When eugenol concentration increased beyond 10 mg L⁻¹, recovery time increased as eugenol concentration increased. Yellow perch exposed to 20 and 30 mg L⁻¹

eugenol at the lightest loading density of 120 g L⁻¹ had the longest recovery time. The overall longest mean recovery time was 76±28.8 min for the yellow perch exposed to 30 mg L⁻¹ eugenol for 6 h at the 120 g L⁻¹ loading density.

Survival – Yellow Perch

The survival of yellow perch was assessed for 7 days following exposure to eugenol (Table 1.7). Mean percent survival was > 95% across all loading densities, durations and eugenol concentrations, with the exception of yellow perch exposed to 20 and 30 mg L⁻¹ eugenol at a 360 g L⁻¹ loading density for 10 h where survival was 84.2±6.0% and 83.6±2.2%, respectively. There was a significant difference in survival for yellow perch held at 360 g L⁻¹ loading density for 10 h ($F_{3,8} = 5.504$, $p < 0.05$). Tukey's HSD post hoc test showed significantly lower survival for yellow perch exposed to 30 mg L⁻¹ eugenol relative to 10 mg L⁻¹ eugenol ($p < 0.05$). No other significant differences in survival were detected ($p > 0.05$).

Behavior and Eugenol Depletion – Tilapia

Eugenol appeared to be removed from the water faster by tilapia than yellow perch. While the longest time for yellow perch to reduce eugenol in buckets to 50% of the starting concentration was 2 h, all tilapia tanks were < 50% of the starting eugenol concentration by 0.5 h, regardless of density (Figure 1.5). Similar to yellow perch, eugenol was removed the fastest when tilapia were held at the highest loading density (480 g L⁻¹) and the slowest rate of eugenol depletion for tilapia was at the lightest density (240 g L⁻¹).

The sedative effects of eugenol on tilapia appear to be minimal (Figure 1.6). Loss of equilibrium increased as eugenol concentration increased and decreased as loading density increased for tilapia. No tilapia lost equilibrium in control or 10 mg L⁻¹ eugenol buckets. The longest loss of equilibrium (approx. 3 h) occurred when tilapia were exposed at combinations of lower loading density (240 and 360 g L⁻¹) and higher eugenol concentration (30 mg L⁻¹). The longest duration of light sedation (3 h) was achieved using 20 mg L⁻¹ eugenol at 480 g L⁻¹ loading density. Tilapia in all treatment tanks exhibited a minimum of 6 h of normal activity regardless of loading density and starting eugenol concentration throughout the entire 10 h exposure. This behavior would be expected given the rapid rate of eugenol depletion from the water (Figure 1.5).

Ammonia – Tilapia

Following a 2, 6 and 10 h eugenol exposure, TAN was measured in all buckets containing tilapia (Figure 1.7). There was a significant main effect of the loading density on the TAN in buckets at 2 h ($F_{2,24} = 9.782$, $p < 0.01$). Tukey's HSD post hoc test revealed that TAN was significant lower for 240 and 360 g L⁻¹ loading densities relative to 480 g L⁻¹ (both p 's < 0.01). Eugenol concentration was also found to be a significant main effect on TAN ($F_{3,24} = 9.404$, $p < 0.01$). The Tukey's HSD post hoc test showed TAN was significantly lower for 0 mg L⁻¹ eugenol buckets relative to all other concentrations tested (all p 's < 0.01). There was a non-significant interaction effect for loading density and eugenol concentration at 2 h ($F_{6,24} = 0.858$, $p > 0.05$).

There was a significant main effect of loading density at 6 h ($F_{2,24} = 12.421$, $p < 0.01$). Tukey's HSD post hoc revealed that TAN was significantly lower for 240 and 360 g L⁻¹ densities relative to the 480 g L⁻¹ density (both p 's < 0.01). A significant main effect of eugenol concentration was found at 6 h ($F_{3,24} = 20.813$, $p < 0.01$). Tukey's HSD post hoc showed the 0 mg L⁻¹ bucket had significantly lower TAN relative to all other eugenol concentrations (all p 's < 0.01). A non-significant interaction effect between loading density and eugenol concentration at 6 h was found ($F_{6,24} = 1.371$, $p > 0.05$).

At 10 h of eugenol exposure, a significant main effect of loading density was found ($F_{2,24} = 5.509$, $p < 0.01$). Tukey's HSD post hoc indicated significantly lower TAN levels for 240 g L⁻¹ relative to the 480 g L⁻¹ loading densities ($p < 0.01$). There were non-significant main effects for eugenol concentration ($F_{3,24} = 1.211$, $p > 0.05$) and non-significant interaction effects between loading density and eugenol concentration on TAN after 10 h of exposure ($F_{6,24} = 0.431$, $p > 0.05$).

Recovery – Tilapia

Recovery was rapid for tilapia when placed in fresh water following eugenol exposure (Table 1.8). Tilapia recovery times were immediate across all treatment combinations, with the exception tilapia exposed to 30 mg L⁻¹ eugenol at 240 and 360 g L⁻¹ loading density for 2 h which had 2.3±1.2 and 1.0±1.0 min recovery times, respectively.

Survival – Tilapia

The survival of tilapia was assessed for 7 days after transfer of tilapia to freshwater subsequent to eugenol exposure (Table 1.9). Unlike yellow perch, mortalities occurred for tilapia at all three eugenol concentrations and in the control. Mean percent survival was > 90% for all loading densities, durations and eugenol concentrations, with the exception of tilapia held at a 240 g L⁻¹ density for 6 h and exposed to 10 mg L⁻¹ eugenol (87.8±2.4% survival). However, Mann Whitney U test showed a non-significant difference in survival between 10 mg L⁻¹ and 0 mg L⁻¹ eugenol ($p > 0.05$) and between 10 mg L⁻¹ and 20 mg L⁻¹ eugenol ($p > 0.05$) for the 240 g L⁻¹ loading density exposed for 6 h. No other significant differences in survival were found at all treatment levels.

DISCUSSION

The live transport of fishes results in high costs incurred by fish farmers and directly impacts potential profit (Pearson et al. 2009). Transport expenses include fuel, labor, oxygen, vehicle maintenance and mortality of fish post-transport. Fish haulers would benefit economically by successfully transporting fish at higher loading densities than the current industry standard. Increasing loading densities would allow fish haulers to make fewer transport trips and minimize transport expenses while increasing the mass of fish transported. To mitigate physiological imbalance and negative conspecific interaction (i.e. aggression, biting and physical injury) experienced at high loading densities, the effects of various additives, including sedatives, to transport tanks on fish mortality has been explored. Although MS-222 is the only FDA-approved sedative in the United States, the sedative AQUI-S®20E (10% eugenol) has received attention in aquaculture due to the potential for a decreased withdrawal time. Because AQUI-S®20E is a relatively new sedative to reach the market, data is needed to assess its effectiveness for animal welfare. My goal was to evaluate the effectiveness of AQUI-S®20E as a sedative for transport purposes through a simulated transport of yellow perch and tilapia fingerlings at elevated loading densities.

Due to the lack of literature where eugenol is the only active ingredient used to sedate fish, many comparisons were made to studies that evaluated clove oil as a potential sedative of fishes. Eugenol is the primary active compound in clove oil (70-90% by weight). However, clove oil also contains low concentrations of the active compounds isoeugenol, acetyl eugenol and other turpenoid compounds. Therefore, it is important to note that clove oil is banned for

commercial use in the United States by the FDA for sedation of fish due to the potential carcinogenic properties of the additional active compounds.

Behavior During AQUI-S®20E Exposure

Although the level and duration of sedation varied with eugenol concentration and loading density, it appeared that AQUI-S® 20E effectively reduced the behavioral activity of yellow perch during simulated transport at loading densities two to three times higher than the industry standard. Aside from potentially decreasing physical injury during transport through collisions with tank walls and conspecifics (Cooke et al. 2004), a reduction in activity has also been found to decrease the physiological stress response of fish (Cubero and Molinero 1997; Chandroo et al. 2005). Stress reduction can lead to increased fillet quality (Rahmanifarah et al. 2011) and higher survival (Hasan and Bart 2007; Iverson et al. 2009), which are important for successful transport. The light sedation I observed suggests that it is likely yellow perch transported in AQUI-S®20E had decreased physiological disturbances relative to fish in tanks that were not sedated.

The concentrations of eugenol tested in this study to induce light sedation were much higher than previously reported for the live transport of fishes. The ideal level of light sedation for transport should allow maintenance of equilibrium while suppressing the flight response to gross external stimuli (Summerfelt and Smith 1990). Yellow perch exposed to 20 and 30 mg L⁻¹ eugenol in this study did display behavior consistent with being lightly sedated for 6 - 8 h. However, other species appeared to be lightly sedated with far less eugenol. For example, cardiac output was reduced and equilibrium was maintained in largemouth bass *Micropterus*

salmoides exposed to 5 – 8.5 mg L⁻¹ clove oil during transport for 2 h at a low loading density of approximately 4 g L⁻¹ (Cooke et al. 2004). Reduced cardiac output suggests that clove oil effectively lightly sedated largemouth bass. The density was also well below the lowest density tested in the current study, which may partly explain why a much lower concentration of clove oil was needed to induce light sedation.

Silver catfish *Rhamdia quelen* showed a significant reduction in ventilatory frequency when exposed to approximately 3.0 mg L⁻¹ eugenol at a loading density of 170 g L⁻¹ (Becker et al. 2012). Reduced ventilatory beats are often used to quantify light sedation during transport (Forgan and Forster 2010) and can be used to infer decreased oxygen consumption and a reduced metabolic rate when exposed to sedatives (Cooke et al. 2004; Chandroo et al. 2005). The concentration of eugenol used to significantly reduce ventilatory frequency in silver catfish was approximately 3 to 10 times lower than the concentrations of eugenol I found effective to induce light sedation in yellow perch. When held at higher loading densities than previously studied, 20 and 30 mg L⁻¹ eugenol was required to induce light sedation for 6 – 8 h in most yellow perch treatment groups.

It is important to note that 20 and 30 mg L⁻¹ eugenol doses resulted in a loss of equilibrium for up to 4 h in most yellow perch treatment groups. The loss of equilibrium during transport is not desired due to the potential for suffocation of fish at the bottom of the transport tank (Summerfelt and Smith 1990). Despite most tanks of yellow perch losing equilibrium at the beginning of the simulated transport, suffocation did not appear to have occurred because high survival rates (>90%) were observed during the 7 day post-transport observation. The lack of suffocation in yellow perch is potentially attributed to the low numbers

of fish in each treatment tank resulting from the small volume of water used in this study. Yellow perch did not appear to cluster at the bottom of the tank and opercular expansion and contraction did not appear to be restricted. Also, fish could have been moved around in the buckets during water quality measurements, which would have also prevented long-term stacking of fish on one another. Finally, the water was saturated with oxygen so any movement of water across the gills likely resulted in oxygen uptake.

In contrast, AQUI-S®20E appears to be effective for a much shorter duration to reduce the behavior of tilapia during simulated transport. Tilapia at all loading densities and eugenol concentrations tested showed normal activity at 4 h during exposure to AQUI-S®20E. Similar to the contrasting behavior I observed between yellow perch and tilapia when exposed to AQUI-S®20E, Hoskonen and Pirhonen (2004) observed behavioral differences between five species of temperate fishes when exposed to 20 mg L⁻¹ clove oil. Induction and recovery from sedation varied between salmonid, percid and cyprinid fish. Unexpectedly, they also observed differences in the sedation properties within the four salmonid species tested. Hence, it was expected that the behavior of yellow perch and tilapia would differ and it is likely that sedation with AQUI-S®20E would vary between most other fish species especially when temperature requirements for the species are substantially different (Hamackova et al. 2001; Hamackova et al. 2004; Woolsey et al. 2004).

As with yellow perch, the eugenol concentrations in the current study were much higher than previous found effective for Nile tilapia. Simoes et al. (2011) found that 9 mg L⁻¹ clove oil resulted in behavior consistent with light sedation of Nile tilapia transported in plastic bags at a loading density of 25 g L⁻¹ for 6 h. In contrast, I observed no signs of sedation for Nile tilapia at 6

h of AQUI-S®20E exposure. The differences in behavior may have resulted from (1) substantially different loading densities (i.e. my densities tested were ten to twenty times higher), (2) different sizes of tilapia tested, (3) additional active compounds found in clove oil, or (4) sealed versus open transport containers. Oxygen available to fish is limited during sealed bag transport, which differs from the steady supply of oxygen diffused into tanks throughout my simulated transport. Regardless, at high loading densities AQUI-S®20E effectively reduced behavior of tilapia for only a short duration (≤ 2 h) during simulated transport. When normal behavior of tilapia returned at 2 h of AQUI-S®20E exposure, eugenol levels in the transport buckets were all below 1-3 mg L⁻¹ eugenol. Thus, it appears that sedation of tilapia only occurred when eugenol concentrations were approximately 2-3 mg L⁻¹ in the transport buckets.

Eugenol Removal From Water

While many studies evaluated behavior when assessing the effectiveness of sedatives for fish transport, this is the first study to report the removal of eugenol from the water by fish. In general, the rate of eugenol removal from the simulated transport tanks increased as loading density increased for both yellow perch and tilapia.

The impact of loading density on the removal of sedatives from the transport tanks is important for expanding previously conducted work using sedatives during fish transport. Previous studies on sedation during transport reported much lower loading densities than the current study (Kaiser et al. 2006; Iverson et al. 2009; Becker et al. 2012). In those studies, fish displayed behaviors consistent with light sedation when exposed to clove oil or eugenol concentrations ranging from approximately 4.0 mg L⁻¹ to 18 mg L⁻¹. The rapid removal of

eugenol from transport tanks in this study could be correlated to the higher density, which also seemed to impact the observed behavior. Therefore, a higher concentration of sedative would likely be needed for those previous studies to achieve the same behavior if a loading density similar to those in this study were used.

Survival 7-Days Post-AQUI-S®20E Exposure

Survival at elevated loading densities was high for yellow perch and tilapia fingerlings 7 day post-transport. Mean survival for yellow perch was > 95% at most loading densities, eugenol concentrations (including control) and durations tested, which suggested that AQUI-S®20E sedation is safe for yellow perch fingerlings. However, significantly lower survival ($83.6 \pm 2.2\%$) of yellow perch occurred when exposed for 10 h to 30 mg L^{-1} eugenol at the highest loading density (360 g L^{-1}). Prolonged exposure to sedatives at high concentrations can result in oxygen debt stemming from reduced oxygen uptake (Guo et al. 1995a; Hill and Forster 2004). This can lead to hypoxemia and ultimately decreased survival following sedation (Sladky et al. 2001). Despite the saturation of oxygen in the buckets, yellow perch at the highest loading density may have built up an oxygen debt if respiration was impaired from sedation (Hill and Forster 2004).

The high survival of Nile tilapia 7 day post-transport with AQUI-S®20E differs from the findings of Simoes et al. (2011) where significantly higher mortality was observed for Nile tilapia transported in 18 mg L^{-1} clove oil relative to a transported control. The tilapia tested by Simoes et al. were roughly 0.5 g and may have been more sensitive to transport and sedation than the larger tilapia ($18.34 \pm 6.91 \text{ g}$) used in this study. Additionally, I intentionally saturated the

dissolved oxygen in the transport buckets while the sealed bag contained a limited amount of dissolved oxygen for the small tilapia. High dissolved oxygen levels can reverse the negative effects of degrading water quality and thereby may have positively contributed to the high survival observed (Randall and Tsui 2002; Harmon 2009). Based on their high mortality, Simoes and colleagues concluded that clove oil should be avoided during transportation of Nile tilapia. However, the high survival and lack of differences in survival between controls and treatment tanks in this study for Nile tilapia suggest that AQUI-S® 20E is safe to use during transport at high densities.

Recovery Times Following AQUI-S® 20E Exposure

Recovery time increased with eugenol concentration and decreased with loading density for yellow perch, which is similar to trends found between eugenol concentration and behavior. Prolonged recovery times in yellow perch exposed to 30 mg L⁻¹ eugenol suggests that central nervous system (CNS) disturbance had occurred. The sedation effect of eugenol may occur because eugenol can act as an agonist on gamma-aminobutyric acid (GABA) receptors. GABA is an inhibitory neurotransmitter that suppresses CNS function (Guenette et al. 2007). Despite seemingly normal behavior observed during the latter duration of exposure to eugenol, some yellow perch required additional time to recover when placed in fresh water (i.e. recovery time >0 min). This suggests that a small amount of CNS disturbance may have persisted post-transport. Further research must be done to determine if GABA receptors are involved.

A dose-dependent recovery time was also observed for common carp *Cyprinus carpio* when sedated with eugenol (Hikasa et al. 1986). The mechanism that prolongs recovery is

thought to be CNS depression from eugenol exposure that results in a decrease in respiratory rates, which in turn reduces metabolism slowing cell functions involved in clearing the sedative (Hill and Forster 2004).

The critical recovery time following sedation should be less than 10 minutes (Marking and Meyer 1985). Although fish exposed to eugenol in other studies have reported recovery times that are longer relative to the current FDA approved MS-222 for sedation to handleable (Hikasa et al. 1986; Sladky et al. 2001; Sink et al. 2007), the majority of yellow perch in this study recovered within 10 min when density was increased above 120 g L^{-1} and eugenol concentration did not exceed 20 mg L^{-1} . Therefore, with recovery below critical times, it appears that AQUI-S®20E may be effective at allowing fish haulers to transport at two to three times higher loading densities using a dose of $\leq 20 \text{ mg L}^{-1}$ eugenol (200 mg L^{-1} AQUI-S®20E). Tilapia recovered rapidly following exposure to AQUI-S®20E at all loading densities. While rapid recovery time is desired following sedation, the duration of reduced behavior was less than for yellow perch. This suggests that sedation of Nile tilapia with AQUI-S®20E may benefit fish haulers primarily during initial loading and transport.

Ammonia Accumulation

AQUI-S®20E was not effective at significantly reducing total ammonia nitrogen (TAN) accumulation in transport buckets. Ammonia is a waste product of metabolism generated through the catabolism of proteins (Guo et al. 1995b). As expected, TAN significantly increased with duration of simulated transport and loading density, similar to results seen in other studies with silver catfish, red porgy *Pagrus pagrus*, and jundia (Golombieski et al. 2003; Pavlidis et al.

2003; Carneiro et al. 2009). I did not see any significant reduction in TAN at any eugenol concentration tested. Therefore, when increasing the loading densities of yellow perch, it is important to understand that ammonia will increase with increased loading densities and eugenol does not appear to mitigate the increase in ammonia accumulation.

While specific toxicity of ammonia on yellow perch is unknown, research has shown that ammonia toxicity in fish decreases when fish are held under higher dissolved oxygen conditions (Randall and Tsui 2002; Treasurer 2010). Given the limitations of my equipment, dissolved oxygen levels were saturated throughout the entire simulated transport and may have positively contributed to my high survival for both species despite increased TAN.

Similar to yellow perch, tilapia showed a significant increase in TAN as loading density increased. I also observed significantly higher TAN levels in buckets containing tilapia exposed to eugenol for 2 and 6 h. However, at 10 h, there was no difference in TAN between eugenol treatments. While tilapia are known to be tolerant to moderately high levels of ammonia (Randall and Tsui 2002), the application of eugenol in transport tanks was not effective at decreasing ammonia levels for tilapia at high loading densities.

CONCLUSION

When using AQUI-S®20E to sedate fish during transport, it is important to consider how species and loading density will impact sedation. Loading density appears to have a negative effect on the level of sedation reached. Additionally, individual species appear to respond differently to sedation. Overall, it appears that loading densities may be increased through sedation with AQUI-S®20E for some species due to decreased conspecific interaction and high observed survival up to 7 days following simulated transport.

Yellow perch exposed to 20 mg L⁻¹ eugenol (200 mg L⁻¹ AQUI-S® 20E) at 240 g L⁻¹ loading density (two times higher than the industry standard) showed behavior indicating light sedation, had quick recovery times and >90% survival for 7 d following a 10 h simulated transport at 17°C. Although additional research is essential to further evaluate AQUI-S®20E for transport, results from this study suggest that AQUI-S®20E may improve the welfare of yellow perch during transport.

Tilapia showed high survival during simulated transport for loading densities up to 480 g L⁻¹, which is two times higher than what is currently recommended. AQUI-S® 20E induced light sedation for 1 -3 h at 20 and 30 mg L⁻¹ eugenol and tilapia immediately recovered when placed in fresh water. However, light sedation did not persist for longer than 3 h and ammonia concentration was not reduced during simulated tilapia transport for up to 10 h in 24°C water. Ammonia concentration was greater in tanks of tilapia exposed to AQUI-S®20E during simulated transport than controls, suggesting that metabolism of protein persisted or was elevated in fish exposed to eugenol. Although AQUI-S®20E did not reduce TAN or induce light sedation for 3 h, AQUI-S® 20E administered at 30 mg L⁻¹ eugenol may be effective to calm

tilapia during the initial phase of transport at loading densities up to 480 g L⁻¹ which may benefit haulers during loading of tilapia into transport tanks.

Future Research

Future research is needed to expand the work completed in this study. The effectiveness of sedation and fish transport are impacted by fish species, fish size, temperature, loading density and duration. To more closely match the numerous biotic and abiotic situations for hauling live fish, it will be important to assess each of these variables and provide additional data to fish haulers. Particularly, fish life stage and temperature are potentially important given the diverse conditions under which fish are transported (i.e. biotic and abiotic factors that are beyond the control of the fish hauler). For example, yellow perch transported in June may be smaller and may be transported at warmer temperatures than yellow perch transported in late October. Fish haulers would greatly benefit from a nomograph to aid in choosing the correct AQUI-S®20E concentration to lightly sedate transported fish.

Although a simulated transport in a laboratory conditions was important for a controlled range finding study, the applicability of this research needs to be examined under actual fish transport conditions. Results from this range finding study should be used to aid in choosing a beneficial loading density and AQUI-S®20E concentration for yellow perch and tilapia. Truck transport will present additional stressors that were not present in a laboratory. Results will provide a comparison to the survival of yellow perch and tilapia post-transport.

Since I discovered substantial differences in sedation between yellow perch and tilapia, it will be important to apply this experimental design to additional species, primarily those of

differing thermal requirements. Salmonids (e.g. rainbow trout, lake trout and brook trout) and ictalurids (e.g. channel catfish) comprise a substantial proportion of the fish transported in the United States for both food fish and stocking into the wild. Additionally, baitfish are routinely hauled daily to bait shops and as forage. Therefore, it would be advised to expand the species tested to include salmonids, ictalurids and baitfish due to their applicability in the U.S. aquaculture industry.

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Table 1.1. Mean (\pm SD) of total length (TL) and wet weight (WT) for yellow perch. Wet weights were measured prior to each replicate and growth was accounted for in loading density calculations.

	Replicate 1	Replicate 2	Replicate 3	Control Replicate	Overall
TL (cm)	8.3 (0.96)	8.4 (0.90)	8.7 (0.95)	9.0 (1.07)	8.5 (1.00)
WT (g)	5.10 (1.82)	5.48 (1.75)	6.05 (2.01)	6.86 (2.43)	5.77 (2.06)

Table 1.2. Mean (\pm SD) total length (TL) and wet weight (WT) for tilapia. Wet weights were measured before each replicate and growth was accounted for in loading density calculations.

	Replicate 1	Replicate 2	Replicate 3	Control Replicate	Overall
TL (cm)	9.12 (0.68)	9.72 (0.82)	10.35 (0.90)	10.31 (1.74)	9.83 (1.14)
WT (g)	14.49 (3.11)	17.08 (4.52)	21.05 (5.70)	22.39 (11.30)	18.34 (6.91)

Table 1.3. Assignment of treatment buckets. In total, 108 buckets were tested. Treatment schedule encompassed all possible combinations of loading density, eugenol concentration, and transport duration for yellow perch.

Tank	Replicate 1			Replicate 2			Replicate 3			Control Replicate		
	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)
1	10	120	2	10	120	2	10	120	2	0	120	2
2	10	120	6	10	120	6	10	120	6	0	120	2
3	10	120	10	10	120	10	10	120	10	0	120	2
4	10	240	2	10	240	2	10	240	2	0	120	6
5	10	240	6	10	240	6	10	240	6	0	120	6
6	10	240	10	10	240	10	10	240	10	0	120	6
7	10	360	2	10	360	2	10	360	2	0	240	2
8	10	360	6	10	360	6	10	360	6	0	240	2
9	10	360	10	10	360	10	10	360	10	0	240	2
10	20	120	2	20	120	2	20	120	2	0	240	6
11	20	120	6	20	120	6	20	120	6	0	240	6
12	20	120	10	20	120	10	20	120	10	0	240	6
13	20	240	2	20	240	2	20	240	2	0	360	2
14	20	240	6	20	240	6	20	240	6	0	360	2
15	20	240	10	20	240	10	20	240	10	0	360	2
16	20	360	2	20	360	2	20	360	2	0	360	6
17	20	360	6	20	360	6	20	360	6	0	360	6
18	20	360	10	20	360	10	20	360	10	0	360	6
19	30	120	2	30	120	2	30	120	2			
20	30	120	6	30	120	6	30	120	6			
21	30	120	10	30	120	10	30	120	10			
22	30	240	2	30	240	2	30	240	2			
23	30	240	6	30	240	6	30	240	6			
24	30	240	10	30	240	10	30	240	10			
25	30	360	2	30	360	2	30	360	2			
26	30	360	6	30	360	6	30	360	6			
27	30	360	10	30	360	10	30	360	10			
28	0	120	10	0	120	10	0	120	10			
29	0	240	10	0	240	10	0	240	10			
30	0	360	10	0	360	10	0	360	10			

Table 1.4. Assignment of treatment buckets. In total, 108 buckets were tested. Treatment schedule encompassed all possible combinations of loading density, eugenol concentration, and transport duration for tilapia.

Tank	Replicate 1			Replicate 2			Replicate 3			Control Replicate		
	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)	Density (g L ⁻¹)	Duration (h)
1	10	240	2	10	240	2	10	240	2	0	240	2
2	10	240	6	10	240	6	10	240	6	0	240	2
3	10	240	10	10	240	10	10	240	10	0	240	2
4	10	360	2	10	360	2	10	360	2	0	240	6
5	10	360	6	10	360	6	10	360	6	0	240	6
6	10	360	10	10	360	10	10	360	10	0	240	6
7	10	480	2	10	480	2	10	480	2	0	360	2
8	10	480	6	10	480	6	10	480	6	0	360	2
9	10	480	10	10	480	10	10	480	10	0	360	2
10	20	240	2	20	240	2	20	240	2	0	360	6
11	20	240	6	20	240	6	20	240	6	0	360	6
12	20	240	10	20	240	10	20	240	10	0	360	6
13	20	360	2	20	360	2	20	360	2	0	480	2
14	20	360	6	20	360	6	20	360	6	0	480	2
15	20	360	10	20	360	10	20	360	10	0	480	2
16	20	480	2	20	480	2	20	480	2	0	480	6
17	20	480	6	20	480	6	20	480	6	0	480	6
18	20	480	10	20	480	10	20	480	10	0	480	6
19	30	240	2	30	240	2	30	240	2			
20	30	240	6	30	240	6	30	240	6			
21	30	240	10	30	240	10	30	240	10			
22	30	360	2	30	360	2	30	360	2			
23	30	360	6	30	360	6	30	360	6			
24	30	360	10	30	360	10	30	360	10			
25	30	480	2	30	480	2	30	480	2			
26	30	480	6	30	480	6	30	480	6			
27	30	480	10	30	480	10	30	480	10			
28	0	240	10	0	240	10	0	240	10			
29	0	360	10	0	360	10	0	360	10			
30	0	480	10	0	480	10	0	480	10			

Table 1.5. Mean (\pm SD) water quality parameters for yellow perch during eugenol exposure and 7 d post-exposure observation. Water quality parameters were measured at 0, 1, 2, 4, 6, 8 and 10 h during static eugenol exposure-and once-daily for the 7 d flow-through post-exposure observation. Data were pooled for all treatment groups and replicates.

	Temperature (°C)	pH	Dissolved Oxygen (mg L ⁻¹)	Flow Rate (mL min ⁻¹)
Eugenol exposure	17.6 (0.16)	7.75 (0.40)	21.8 (1.76)	NA
7-d Post-exposure	17.7 (0.07)	8.10 (0.10)	8.60 (0.42)	262 (22.9)

Table 1.6. Mean (\pm SD) water quality parameters for tilapia during eugenol exposure and 7 d post-exposure observation. Water quality parameters were measured at 0, 1, 2, 4, 6, 8 and 10 h during static eugenol exposure-and once-daily for the 7 d flow-through post-exposure observation. Data were pooled for all treatment groups and replicates.

	Temperature (°C)	pH	Dissolved Oxygen (mg L ⁻¹)	Flow Rate (mL min ⁻¹)
Eugenol exposure	21.4 (0.38)	7.78 (0.40)	21.8 (1.12)	NA
Post-exposure	22.1 (0.2)	7.84 (0.11)	6.36 (0.94)	269 (21.1)

Table 1.7. Mean (\pm SEM) percent survival for yellow perch 7 d following exposure to eugenol.

Treatments with different letters are significantly different from each other ($p < 0.05$).

Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)			
		Control	10	20	30
120	2	100 (0)	100 (0)	100 (0)	100 (0)
240	2	100 (0)	100 (0)	100 (0)	99.1 (0.9)
360	2	100 (0)	100 (0)	100 (0)	100 (0)
120	6	100 (0)	100 (0)	100 (0)	100 (0)
240	6	100 (0)	99.1 (0.9)	100 (0)	99.1 (0.9)
360	6	100 (0)	99.4 (1.6)	100 (0)	96.5 (1.9)
120	10	100 (0)	100 (0)	98.1 (1.9)	100 (0)
240	10	100 (0)	98.1 (1.9)	99.1 (0.9)	98.1 (0.9)
360	10	^{ab} 95.3 (1.4)	^a 98.8 (1.2)	^{ab} 84.2 (6.0)	^b 83.6 (2.2)

Table 1.8. Summary of mean (\pm SEM) recovery times (min) for tilapia placed in flow-through fresh water immediately after transfer from static exposure to eugenol. Recovery from sedation was defined as upright, active swimming and the absence of any signs of sedation for 100% of fish within the tank. Zero minutes represents immediate recovery when placed in fresh water.

Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)			
		Control	10	20	30
240	2	0	0	0	2.3 (1.2)
360	2	0	0	0	1.0 (1.0)
480	2	0	0	0	0
240	6	0	0	0	0
360	6	0	0	0	0
480	6	0	0	0	0
240	10	0	0	0	0
360	10	0	0	0	0
480	10	0	0	0	0

Table 1.9. Mean (\pm SEM) survival (%), for tilapia 7 d following exposure to eugenol. No significant differences were detected.

Density (g L ⁻¹)	Duration (h)	Eugenol (mg L ⁻¹)			
		0	10	20	30
240	2	97.0 (3.0)	94.1 (5.9)	91.7 (4.8)	97.2 (2.8)
360	2	97.9 (2.1)	100 (0)	98.2 (1.9)	100 (0)
480	2	98.4 (1.6)	100 (0)	95.8 (2.4)	98.6 (1.4)
240	6	100 (0)	87.8 (2.4)	93.3 (3.5)	100 (0)
360	6	97.9 (2.1)	100 (0)	96.3 (3.7)	96.3 (3.7)
480	6	98.4 (1.6)	98.6 (1.4)	94.4 (5.6)	97.6 (1.2)
240	10	98.0 (2.0)	95.3 (2.5)	93.3 (3.5)	91.0 (4.6)
360	10	100 (0)	98.2 (1.9)	100 (0)	100 (0)
480	10	97.2 (2.8)	100 (0)	98.6 (1.4)	100 (0)

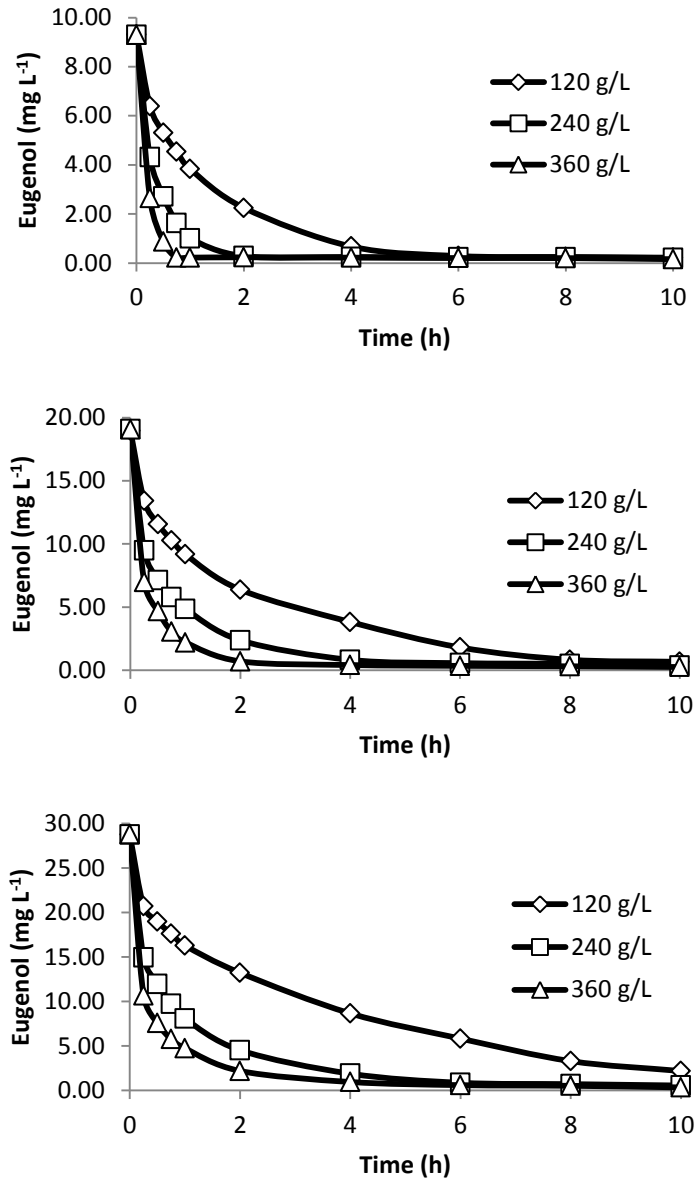


Figure 1.1. Eugenol removal from simulated transport buckets when yellow perch were exposed to 10 (top), 20 (middle) or 30 (bottom) mg L⁻¹ eugenol at 120, 240 and 360 g L⁻¹ loading densities. Water samples were analyzed with HPLC (Agilent HPLC System Model: 1200 Series) over 10 h during eugenol exposure.

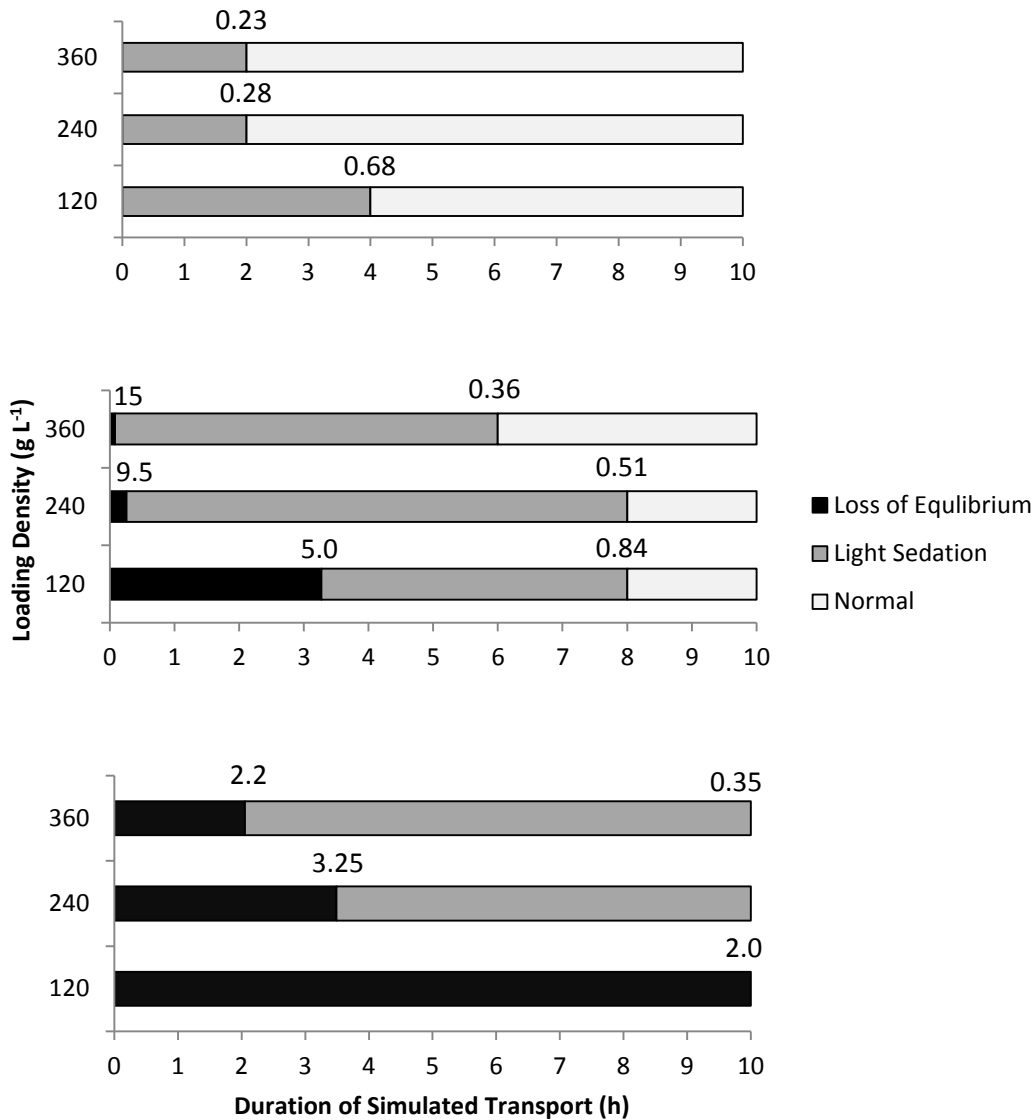


Figure 1.2. Summary of yellow perch behavior throughout 10 h of static exposure to 10 (top), 20 (middle) or 30 (bottom) mg L⁻¹ eugenol. Loss of equilibrium was quantified as > 20% of the fish displaying this behavior. Light sedation was defined as a calculated ET80 of ≤ 20% showing loss of equilibrium and lacking a flight response to hand movement over tank and/or net avoidance. Normal was defined as an observable physical response to an external stimulus and net avoidance. Eugenol concentration (mg L⁻¹) in tanks during behavioral transition was reported above bars.

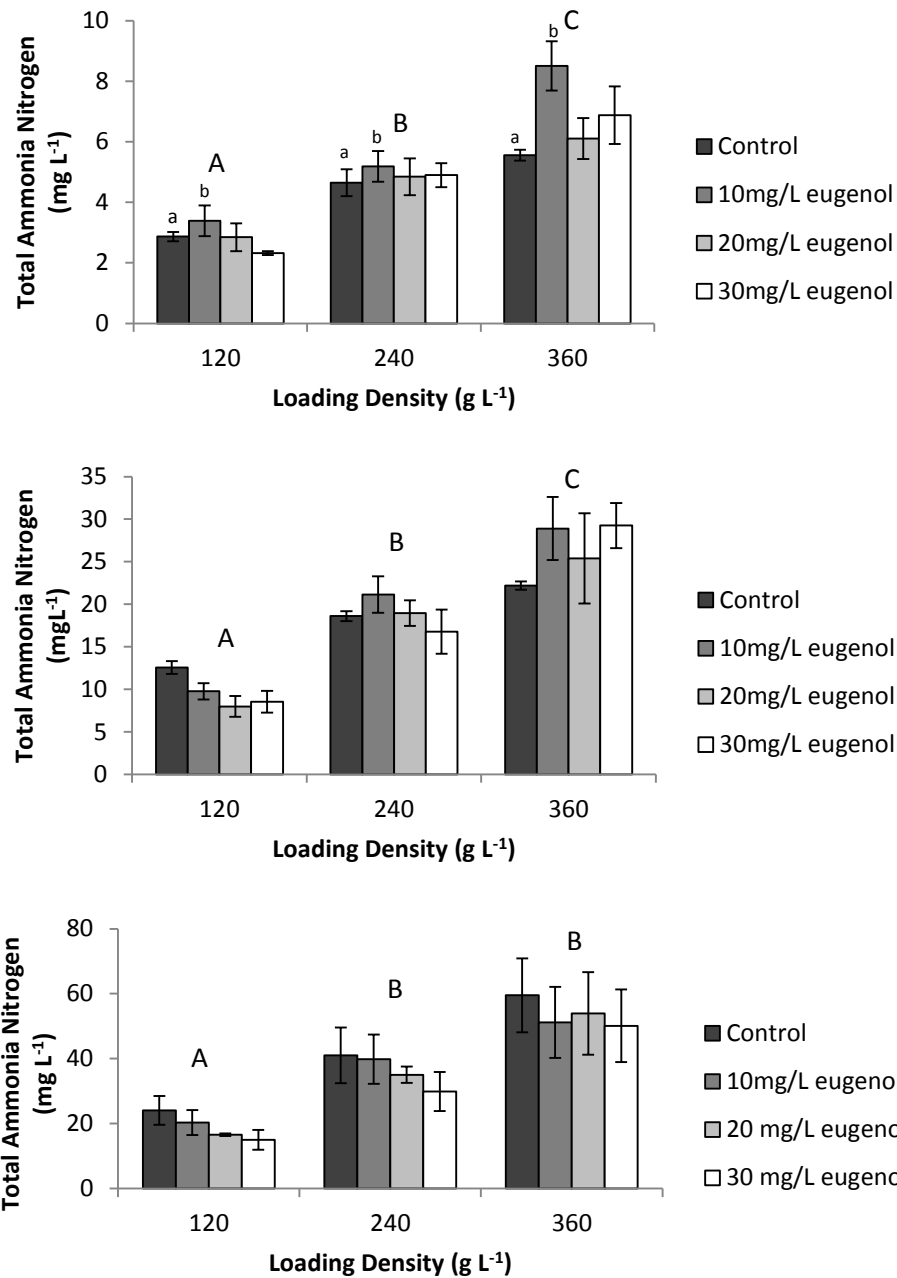


Figure 1.3. Mean (\pm SEM) total ammonia nitrogen (mg L^{-1}) for yellow perch exposed to 0, 10, 20 and 30 mg L^{-1} eugenol for 2 (top), 6 (middle) or 10 h (bottom) at 120, 240 and 360 g L^{-1} loading densities. Different uppercase letters show significant main effects between loading densities and different lowercase letters show significant main effects between eugenol concentrations within each loading density on TAN ($p < 0.05$). No interactions were found.

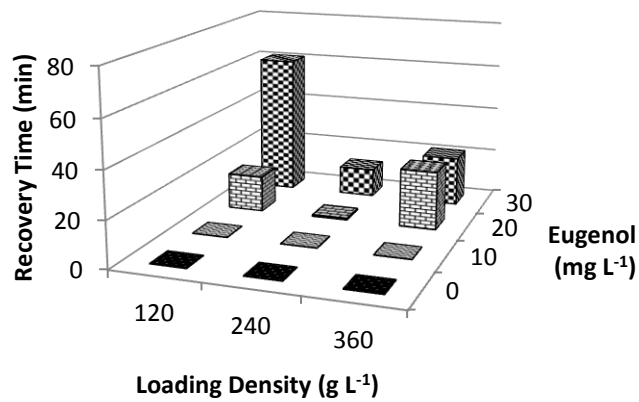
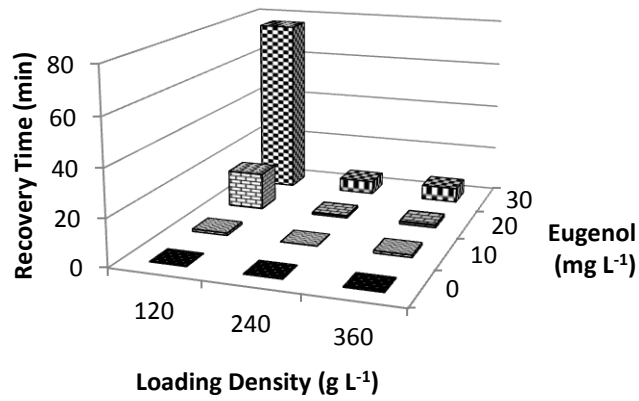
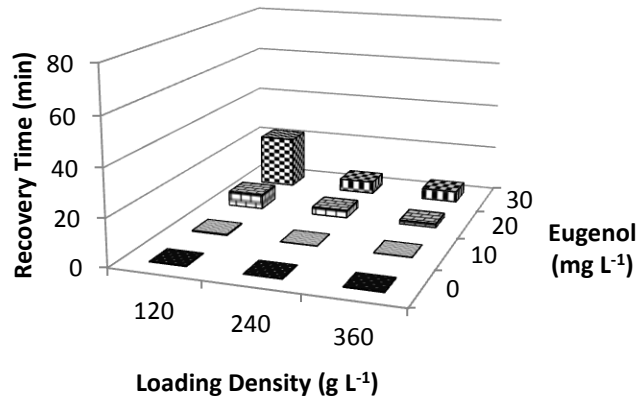


Figure 1.4. Summary of mean recovery times (min) for yellow perch placed in fresh water immediately following exposure to eugenol for 2 (top), 6 (middle) and 10 h (bottom). Recovery from sedation was defined as upright, active swimming and the absence of any signs of sedation for 100% of fish within the tank.

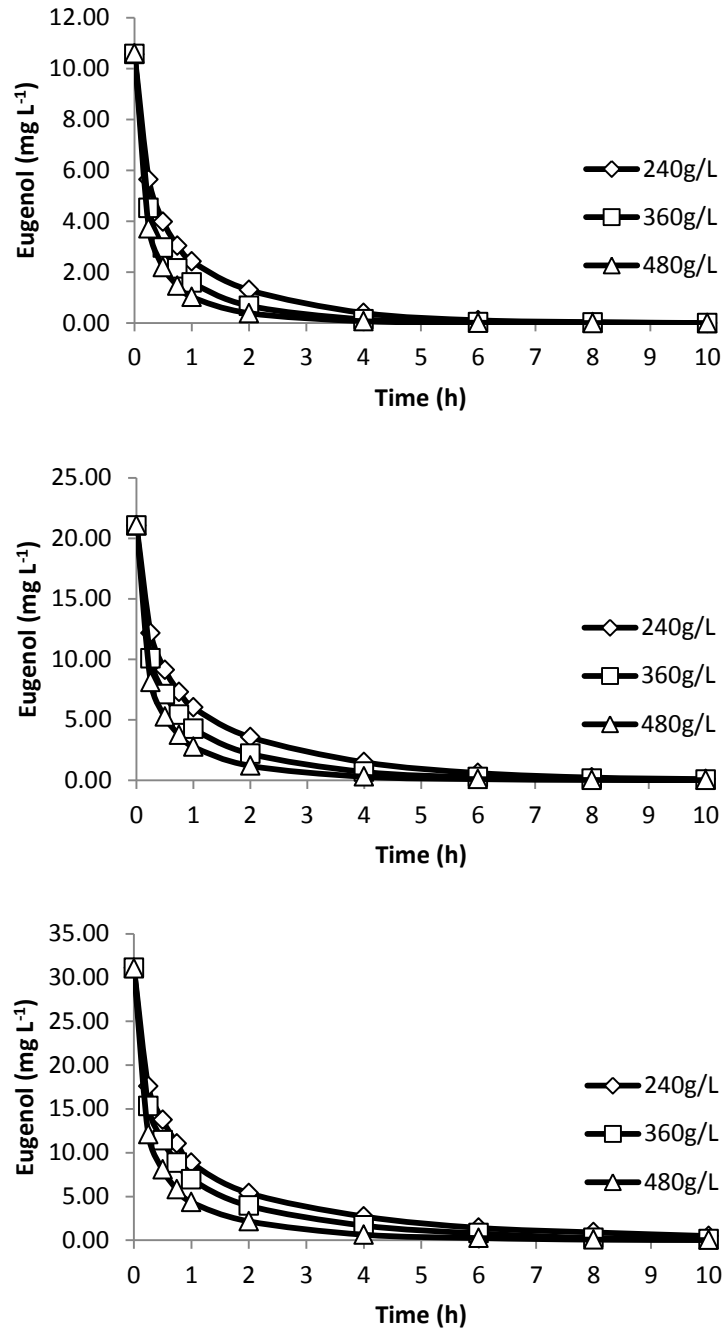


Figure 1.5. Eugenol removal from simulated transport buckets when tilapia were exposed to 10 (top), 20 (middle) or 30 (bottom) mg L⁻¹ eugenol at 240, 360 and 480 g L⁻¹ loading densities. Water samples were analyzed with HPLC (Agilent HPLC System Model: 1200 Series) over 10 h during eugenol exposure.

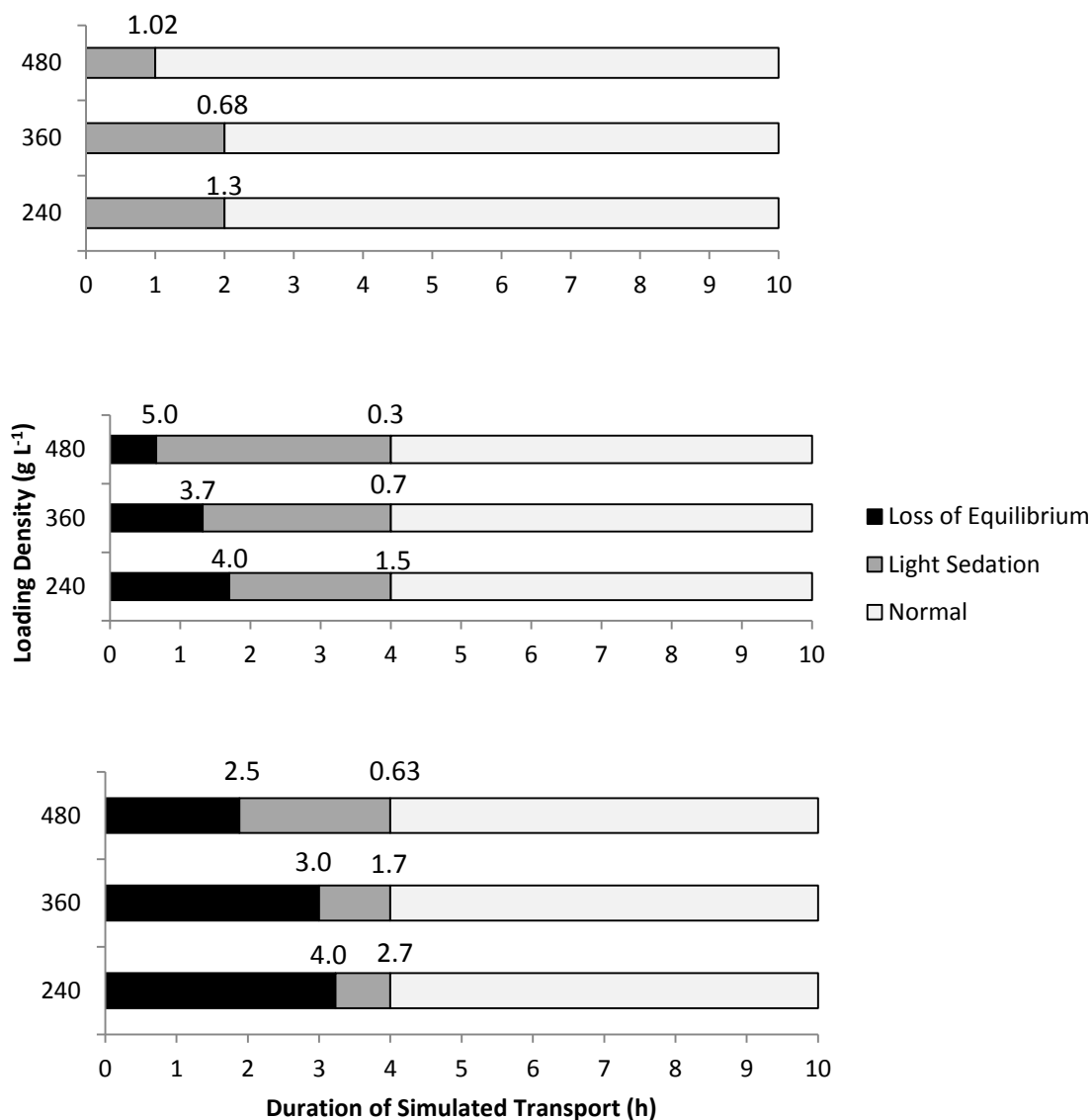


Figure 1.6. Summary of tilapia behavior throughout 10 h of static exposure to 10 (top), 20 (middle) or 30 (bottom) mg L⁻¹ eugenol. Loss of equilibrium was quantified as > 20% of the fish displaying this behavior. Light sedation was defined as a calculated ET80 of ≤ 20% showing loss of equilibrium and lacking a flight response to hand movement over tank and/or net avoidance. Normal was defined as an observable physical response to an external stimulus and net avoidance. Eugenol concentration (mg L⁻¹) in tanks at behavioral transitions was reported above bars.

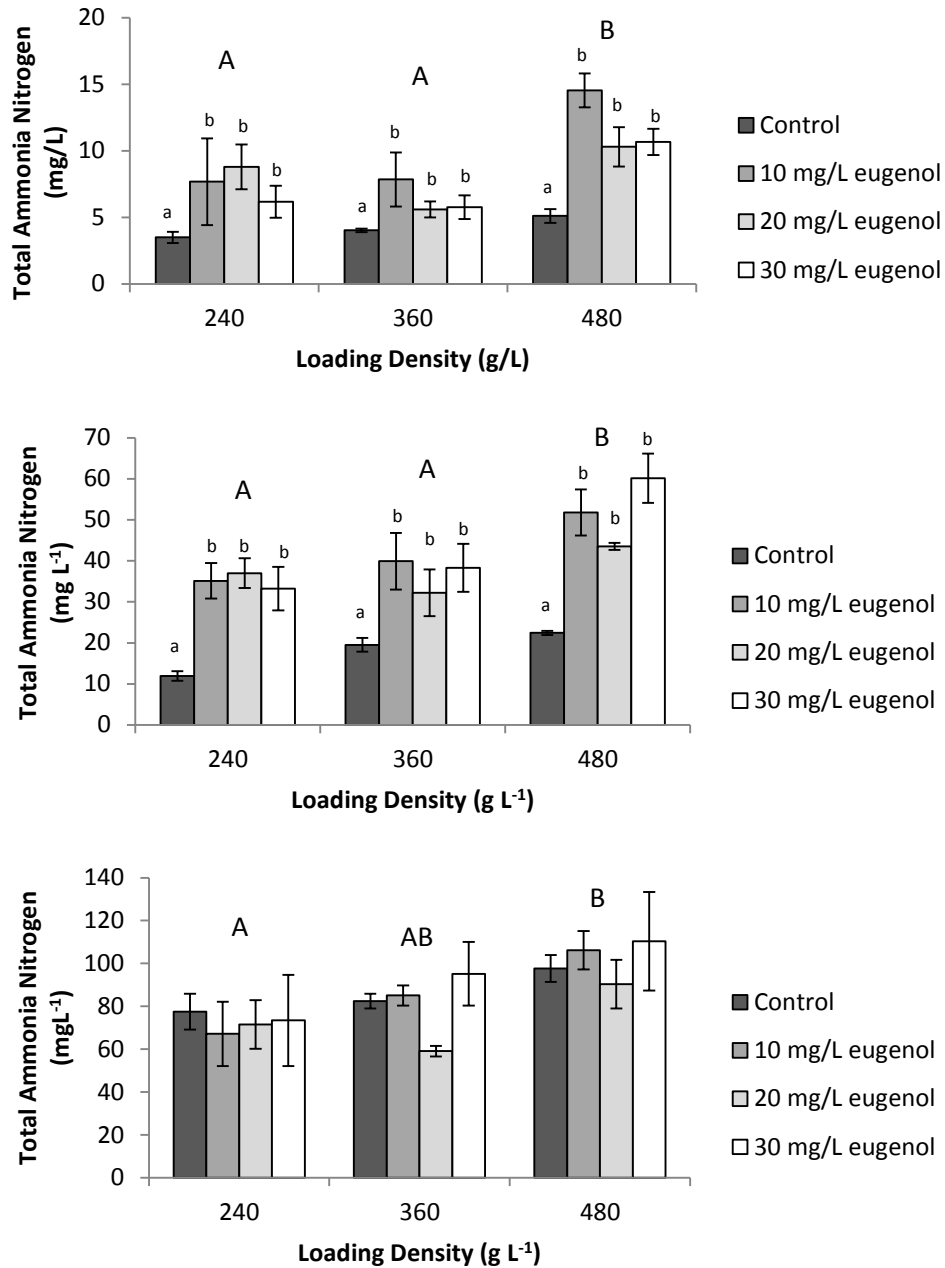


Figure 1.7. Mean (\pm SEM) total ammonia nitrogen (mg L^{-1}) for tilapia exposed to 0, 10, 20 and 30 mg L^{-1} eugenol for 2 (top), 6 (middle) or 10 h (bottom) at 240, 360 and 480 g L^{-1} loading densities. Different uppercase letters show significant main effects between loading densities and different lowercase letters show significant main effects between eugenol concentrations within each loading density on TAN ($p < 0.05$). No significant interactions were found.

CHAPTER 2. RESPIROMETRY

ABSTRACT

Fish transport costs are a substantial portion of the operational expenses of the aquaculture industry, especially as fuel costs continue to rise. Increasing fish loading density during transport could reduce expenses by enabling the transport of more fish mass per gallon of fuel. I hypothesized that the addition of sedatives could reduce metabolic rates at increased loading densities. In this study, yellow perch *Perca flavescens* and Nile tilapia *Oreochromis niloticus* were model species used to examine the effectiveness of AQUI-S®20E (10% eugenol, Lower Hutt, New Zealand) to reduce metabolic rates. AQUI-S®20E is currently under evaluation by the U.S. Food and Drug Administration Center for Veterinary Medicine as an immediate release finfish sedative. Both species were exposed to 0 to 300 mg L⁻¹ AQUI-S®20E (0 to 30 mg L⁻¹ eugenol) at loading densities up to two times the current industry standard during static respirometry. Under loading densities of 59.5±2.3 to 243.4±6.9 g L⁻¹, yellow perch controls (0 mg L⁻¹ eugenol) had metabolic rates of 329.6 – 400.0 mg O₂ kg⁻¹ h⁻¹, while yellow perch exposed to 200 and 300 mg L⁻¹ AQUI-S®20E (20 and 30 mg L⁻¹ eugenol) had metabolic rates of 258.4 – 325.6 and 189.1 – 271.0 mg O₂ kg⁻¹ h⁻¹, respectively. Therefore, concentrations of AQUI-S®20E ranging from 200 to 300 mg L⁻¹ (20 to 30 mg L⁻¹ eugenol) were effective at reducing metabolic rates for yellow perch in 17°C water relative to the unsedated control. Tilapia exposed to 300 mg L⁻¹ AQUI-S®20E (30 mg L⁻¹ eugenol) at 22°C had significantly reduced metabolic rates (424.5±42.3 mg O₂ kg⁻¹ h⁻¹) relative to the control (546.6±53.5 mg O₂ kg⁻¹ h⁻¹) at a loading density of 116.2±7.0 g L⁻¹. No significant differences in metabolic rates for tilapia exposed to 0 – 300 mg L⁻¹ AQUI-S®20E at 215.6±10.2 or 309.6±13.7 g L⁻¹ loading densities. Results indicate that

AQUI-S[®]20E sedation may benefit yellow perch at high densities during transport due to a reduction in metabolic rates, while further research is needed to assess the benefits of AQUI-S[®]20E sedation for tilapia at densities greater than 120 g L⁻¹.

INTRODUCTION

Minimizing metabolic disturbance during fish transport can be challenging for fish culturists and is important for the survival of fish post-transport (Harmon 2009). Respirometry systems are often used to quantify metabolic rate of fish; metabolic rate can serve as an indicator for the quality of the transport environment (Cech 1990). Specifically, changes in metabolic rate can indicate degrading water quality, increased stress and physical injury. Fish metabolism is typically measured through oxygen consumed at rest or during routine or active swimming (Beamish 1963).

Anatomical characteristics of the gills of fish allow efficient removal of dissolved oxygen from water (Ballintijn 1972). Four gill arches lay beneath the hard, outer opercula on each lateral side of the head. Each gill arch contains an anterior bony arch and feathery posterior filaments called lamellae. While the bony arch acts to aid in feeding by filtering or gripping prey, the primary function of the filaments are to serve as sites for gas exchange (Sardet et al. 1979; Evans et al. 1999). Gas exchange, specifically oxygen uptake, occurs through diffusion across the thin epithelial membrane of the lamellae (Hughes 1970). Oxygen uptake by the gills is accomplished through a counter-current exchange system (Hughes 1972; Evans et al. 1999). Oxygen-rich water rushes over the gill surface during ventilation and oxygen-poor blood flows in the opposite direction to water flow. This counter-current exchange allows fish to maximize their oxygen uptake through continuous diffusion of oxygen from water into the blood (Hughes 1972).

During fish transport, oxygen consumption can increase due to stress associated with tank loading (Wedemeyer 1972), high hauling densities (De Abreu et al. 2008) and decreased

water quality (Hill and Forster 2004; Carneiro et al. 2009). Increased oxygen consumption is a secondary stress response in fish that results from increased release of cortisol primary response to physical or chemical stressors (Barton and Iwama 1991; Cho and Heath 2000; Portz et al. 2006). When fish undergo chronic or multiple exposures to a stressor, it can result in a compromised immune system and ultimately mortality (Wendelaar 1997). Mortality resulting from transport stress can persist for up to 14 days post-transport (Ross and Ross 2008).

To mitigate stressors, sedatives have been studied as an additive during transport of live fishes (Cooke et al. 2004; Ross and Ross 2008). Currently, tricaine-methanesulfonate (MS-222) is the only sedative approved for finfish use in the United States. MS-222, an efficacious immobilizing and stress reducing anesthetic (Pramod et al. 2010; Gholipour et al. 2011), is restricted by the 21-day withdrawal period assigned by the U.S. Food and Drug Administration (FDA). Therefore, no human consumption of fish sedated with MS-222 may occur within 21-days post-sedation. For fish transport, there is a strong need for an immediate-release or short (<1-d) withdrawal sedative that can be used in aquaculture and by fisheries managers (Bowker and Trushenski 2012). AQUI-S®20E (10% active eugenol, Lower Hutt, New Zealand) is currently being evaluated by FDA as an immediate-release sedative.

Eugenol, 2-methoxy-4-prop-2-enyl-phenol, is the primary active ingredient in clove oil (70-90% by weight) and has been heavily researched as a general sedative (Hoskonen and Pirhonen 2004; Roubach et al. 2005; Palic et al. 2006; Park et al. 2009). Results of various studies using eugenol or clove oil have shown rapid induction and recovery times (Anderson et al. 1997; Gomes et al. 2011), low physiological disturbances (Cho and Heath 2000), rapid absorption and elimination from tissues (Kildea et al. 2004; Guenette et al. 2007) and the ability

to minimize primary and secondary stress responses (Iverson et al. 2003; Deriggi et al. 2006; Palic et al. 2006). Recent studies by Meinertz et al. (2012, 2013) using the product AQUI-S®20E characterized the depletion of eugenol from rainbow trout tissues. Results of those studies indicated that eugenol quickly depleted from the tissues of rainbow trout at 17°C. Although eugenol has been evaluated for multiple properties of sedation, none of those studies have assessed the effects of AQUI-S®20E on metabolic rates of fish held at high loading densities.

Yellow perch *Perca flavescens* and tilapia *Oreochromis niloticus* are two commercially and economically important fish in the United States (Malison 2000; El-Sayed 2006). Yellow perch are a cool water species cultured for both food fish and for stocking (i.e. recreational harvest). Nile tilapia, a resilient warm water fish generating over \$3.7 billion in aquaculture production worldwide (FAO 2009), has drawn interest as a food fish in the US. While yellow perch can survive short periods of moderate to low dissolved oxygen (3.5 mg L^{-1}), prolonged periods of low dissolved oxygen results in mortality (Malison 2000). Tilapia are more robust to low dissolved oxygen environments, which has promoted their increase in culture worldwide (Abdel Magid and Babiker 1975).

The objective of this study was to examine the effectiveness of AQUI-S®20E to reduce the metabolic rates of yellow perch and Nile tilapia at high loading densities. Specifically, I was interested in identifying an AQUI-S®20E concentration that would decrease metabolic rate of yellow perch and tilapia and potentially allow haulers to increase loading density beyond current industry standards.

MATERIALS AND METHODS

Study Animals

Yellow perch [9.74 ± 4.49 g wet weight, 10.1 ± 1.26 cm total length (TL)] were hatched and reared in 17°C flow-through tanks at the Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI. Nile tilapia (25.91 ± 7.64 g wet weight, 11.4 ± 1.25 cm TL) were obtained from Aquasafra Inc. (Bradenton, FL) as phenotypic males (>80% phenotypic males resulting from feed administered with 17-methyltestosterone) then reared at UMESC in 24°C flow-through tanks. Dissolved oxygen was measured during acclimation and grow-out phases. After the grow-out phase at UMESC, yellow perch and tilapia were then transported to the aquaculture facility at the University of Wisconsin – Stevens Point (Stevens Point, WI). Yellow perch were held in 2800 L and tilapia in 1890 L recirculating aquaculture systems and fed daily maintenance rations of approximately 1% body weight (BW) day⁻¹. Yellow perch were fed Skretting extruded slow-sinking crumbles 1.6 mm salmon diet (Skretting USA, Tooele, UT) and tilapia were fed Purina Aquamax Grower 3/32 in. extruded pellet (Purina Mills, USA). Fish were not fed 12 – 15 h before oxygen consumption measurements.

Respirometry Design

A 1.0-L Erlenmeyer flask was used as a static respirometry chamber and was the experimental unit to measure oxygen consumption. After the flask was filled to capacity with 0, 10, 20 or 30 mg L⁻¹ eugenol (0, 100, 200 or 300 mg L⁻¹ AQUI-S®20E) and fish were added, temperature and dissolved oxygen probes (YSI Model 5350) were inserted into a rubber stopper and placed at the top of the flask to seal it from the atmosphere. Inside the flask was a

2.5 cm magnetic stir bar. The flask was placed on top of a Thermolyne Cimarec®2 Model S46725 stir plate and set to a slow, steady spin. Probes were connected to YSI Model 5300 Biological Oxygen Monitor and a HOBO® 4-Channel External data logger. The data logger recorded temperature and percent dissolved oxygen saturation every 15 s for yellow perch and every 10 s for tilapia.

Loading Density Calculations

Multiple loading densities of yellow perch and tilapia were exposed to AQUI-S®20E to determine its effects on metabolic rates. The sealed respirometry chamber held 1.1 L of water. Both fish species displaced approximately 1.0 mL of water per gram of BW. The total flask volume of 1.1 L and displacement of 1.0 mL g⁻¹ BW were accounted for in loading density calculations. Initially, yellow perch were to be tested at loading densities of 120, 240 and 360 g L⁻¹ and tilapia at 240, 360 and 480 g L⁻¹. Due to the conical shape of the flask, it was decided during pilot trials that yellow perch at 360 g L⁻¹ and tilapia at 480 g L⁻¹ would be an unreasonable density to fit inside the chamber. Therefore, the highest density for each fish was replaced with a lower density and yellow perch were tested at target loading densities of 60, 120 and 240 g L⁻¹ and tilapia at 120, 240 and 360 g L⁻¹. Fish weights were later used to calculate the actual loading densities tested.

To further explore the effects of AQUI-S®20E beyond the three previous loading densities, an additional lower loading density consisting of only two fish per trial was run. The results of this additional loading density were important for assessing the effects of AQUI-S®20E on metabolic rates of both species for fish haulers that haul at low loading densities.

AQUI-S®20E Calculation

The mass of AQUI-S®20E required to make the stock solutions was calculated using the following equation (e.g. 10 mg L⁻¹ eugenol = 100 mg L⁻¹ AQUI-S®20E):

$$A = [(B * C) / 10^3] / 10\% \text{ active eugenol}$$

$$A = \text{AQUI-S®20E (g)}$$

$$B = \text{Volume of stock solution (L)}$$

$$C = \text{Target eugenol concentration (mg L}^{-1}\text{)}$$

Respirometry Trials

AQUI-S®20E was weighed directly into a 50-mL beaker (Mettler Toledo Model AB265-S/FACT balance). All stock solutions were prepared by adding a known AQUI-S®20E mass to 10 L of water from the RAS tank followed by hand mixing for 1 min. Stock solutions of 0, 10, 20 and 30 mg L⁻¹ eugenol were individually prepared and eugenol concentration was not verified. A YSI (Model 63) handheld pH meter was used to measure pH of the stock solution.

Stock solutions were used to fill the respirometry chamber. Blown air was supplied directly into the chamber using a Penn-Plax Silent-AirX4™ pump with a terminal air diffuser. Fish were group weighed into different loading densities (Ohaus Explorer® Model EX4202 balance). Air was bubbled into the respirometry chamber until dissolved oxygen readings stabilized near 100% saturation. Once stabilized, the air diffuser was removed and fish were added to the chamber. The rubber stopper containing the temperature and dissolved oxygen probes was placed in the flask and all air bubbles in the chamber were removed. The HOBO® 4-Channel External data logger (ONSET Inc., Bourne, MA) recorded temperature and percent

saturation outputs from the YSI Model 5300 Biological Oxygen Monitor (Xylem Inc., Yellow Springs, OH) and fish remained in the chamber until dissolved oxygen dropped to approximately 50-60% saturation, which ranged from 2 – 12 min depending on treatment.

Wet weight (g) and TL (cm) were recorded for all fish following respiration measurements. Wet weights for each treatment group were added together for use in mass-specific metabolic rate calculations. Survival was not assessed post-exposure, however all fish were alive when the final wet weight and TL were measured.

Metabolic Rate Calculation

Mass-specific metabolic rates were calculated for each species of fish and for each combination of loading density and eugenol concentration (Cech 1990). Percent saturation of oxygen was converted to mg L⁻¹ dissolved oxygen prior to calculating metabolic rates (USGS 2011). Calculations were conducted as follows:

$$MR = [(DO_{\text{initial}} - DO_{\text{final}}) \times \text{Volume}] / (\text{Time} \times \text{Mass})$$

Where:

MR = metabolic rate (mg O₂ kg⁻¹ h⁻¹)

DO_{initial} = oxygen concentration at an early point in experiment (mg L⁻¹)

DO_{final} = oxygen concentration at a late point in the experiment (mg L⁻¹)

Volume = capacity of chamber (L)

Time = length of time between initial and final (h)

Mass = mass of fish (kg)

Statistical Analyses

Data in tables were reported as mean \pm standard deviation (SD). Data presented graphically were expressed as mean \pm standard error of the mean (SEM). Homogeneity of variances was tested using Bartlett's test. Normality of data was tested using the Shapiro-Wilk test. A log-transformation was used to normalize data when original data were not normally distributed. A one-way ANOVA and Tukey's HSD post hoc test were used to test for significant differences in metabolic rates for each loading density and eugenol concentration. In cases where transformations did not normalize the data, the non-parametric Kruskal-Wallis test was used to test significance. Analyses were performed using R (R Core Team 2013; 64-bit, version 2.15.3) with the R Commander navigational interface (Fox 2005) and a minimum significance level of $P < 0.05$.

RESULTS

The mean wet weight, TL and water quality parameters (temperature and pH) are listed in Table 2.1. Fish weights from each treatment group were used to calculate the loading densities of each replicate (Table 2.2).

Yellow Perch Density

The three loading densities were tested independently at four eugenol concentrations (Figure 2.1). Significant differences in metabolic rates were detected for yellow perch exposed to 0, 10, 20 and 30 mg L⁻¹ eugenol at each of the loading densities (60 g L⁻¹: F_{3,8} = 11.44, p < 0.01; 120 g L⁻¹: F_{3,8} = 16.22, p < 0.01; and 240 g L⁻¹: F_{3,8} = 14.93, p < 0.01). Yellow perch exposed to 30 mg L⁻¹ eugenol had significantly lower metabolic rates than the yellow perch exposed to 0 and 10 mg L⁻¹ eugenol at the 60 g L⁻¹ loading density (Tukey's HSD, p<0.05). Yellow perch exposed to 20 mg L⁻¹ eugenol at the 60 g L⁻¹ loading density showed lower mean metabolic rates than the 0 and 10 mg L⁻¹ eugenol exposures, but the difference was not statistically significant (p>0.05). Metabolic rates of yellow perch at the 120 g L⁻¹ loading density were significantly lower for the 0, 20 and 30 mg L⁻¹ eugenol exposures relative to the 10 mg L⁻¹ exposure (Tukey's HSD, p<0.05). The 240 g L⁻¹ loading density had significantly lower metabolic rates when exposed to 30 mg L⁻¹ eugenol relative to yellow perch exposed to 0, 10 and 20 mg L⁻¹ eugenol (Tukey's HSD, p<0.05). Overall, yellow perch exposed to 20 and 30 mg L⁻¹ eugenol had lower mean metabolic rates than yellow perch exposed to 0 and 10 mg L⁻¹ eugenol at all three loading densities.

An additional comparison was conducted to determine the effect of loading density on metabolic rate at each eugenol concentration (Figure 2.2). Significant differences among loading densities were detected for yellow perch exposed to 30 mg L⁻¹ eugenol ($F_{2,6} = 7.613$, $p < 0.05$). Yellow perch exposed to 30 mg L⁻¹ eugenol at the 120 g L⁻¹ loading density had significantly higher metabolic rates than yellow perch exposed to 30 mg L⁻¹ eugenol at the 60 and 240 g L⁻¹ loading densities (Tukey's HSD, $p < 0.05$). No significant differences in metabolic rates were detected for yellow perch exposed to 0 ($F_{2,6} = 1.748$, $p > 0.05$), 10 ($F_{2,6} = 0.349$, $p > 0.05$) and 20 ($F_{2,6} = 2.21$, $p > 0.05$) mg L⁻¹ eugenol.

Tilapia Density

The metabolic rates for tilapia exposed to 0, 10, 20 and 30 mg L⁻¹ eugenol at the three loading densities are shown in Figure 2.1. Metabolic rates for tilapia exposed to 30 mg L⁻¹ eugenol at the 120 g L⁻¹ loading density were significantly lower than the metabolic rates of tilapia exposed to 0 mg L⁻¹ eugenol at the 120 g L⁻¹ density ($F_{3,8} = 5.764$, $p < 0.05$). No other significant differences in metabolic rates were observed at the 240 ($F_{3,8} = 3.292$, $p > 0.05$) or 360 g L⁻¹ (Kruskal-Wallis $\chi^2 = 5$, $p > 0.05$) loading densities when exposed to all four concentrations of eugenol.

Metabolic rates for tilapia were compared at each eugenol concentration (Figure 2.2). No significant differences in metabolic rates were detected between the three loading densities at 0 ($F_{2,6} = 4.488$, $p > 0.05$), 10 ($F_{2,6} = 3.076$, $p > 0.05$), 20 ($F_{2,6} = 0.061$, $p > 0.05$) and 30 ($F_{2,6} = 1.99$, $p > 0.05$) mg L⁻¹ eugenol.

Yellow Perch – Two Fish

Yellow perch exposed to eugenol at the two fish loading density (Figure 2.3) resulted in significant differences in metabolic rates ($F_{3,8} = 7.743$, $p < 0.01$). The metabolic rate for yellow perch exposed to 30 mg L^{-1} eugenol was significantly lower than the 0 and 10 mg L^{-1} eugenol treatments (Tukey's HSD, $p < 0.05$). The 20 mg L^{-1} eugenol treatment resulted in a decreased mean metabolic rate, but the decrease was not significantly lower than the 0 and 10 mg L^{-1} eugenol exposures (Tukey's HSD, $p > 0.05$).

Tilapia – Two Fish

Significantly reduced metabolic rates ($F_{3,8} = 10.88$, $p < 0.01$) were observed for tilapia exposed to 30 mg L^{-1} relative to 0, 20 and 20 mg L^{-1} eugenol exposed tilapia (Tukey's HSD, $p < 0.05$). The 20 mg L^{-1} eugenol exposure resulted in a decreased metabolic rate relative to the 0 and 10 mg L^{-1} eugenol exposures, but the decrease was not statistically significant (Tukey's HSD, $p > 0.05$).

DISCUSSION

Respirometry experiments in aquaculture are important indicators to understand how fish respond to changes in their rearing environment (Cech 1990). During live transport, fish are subjected to a confined environment for potentially long durations (>1 h) at loading densities greater than typically encountered in most rearing systems (Ross and Ross 2008). Carmichael et al. (2001) suggested in their guidelines for live fish transport that metabolic rate increases should be minimized during transport. Collectively, live transport of fish exposes the fish to inadvertent stressors (Harmon 2009; Iverson et al. 2009) that can negatively impact post-transport survival (Kaiser and Vine 1998; Pearson et al. 2009). Fish transporters would benefit, both in efficiency and economically, from hauling fish at loading densities higher than what are currently used in the industry. The objective for the current study was to assess the effectiveness of AQUI-S®20E to decrease metabolic rates of yellow perch and tilapia when at loading densities higher than those typically used for yellow perch or tilapia transport (Carmichael et al. 2001; Timmons and Ebeling 2007).

Yellow Perch

Yellow perch fingerlings are routinely transported at loading densities as high as 60 g L⁻¹ (Carmichael et al. 2001). The presence of sufficient dissolved oxygen during transport is a primary determinant in the success of live-transport and dissolved oxygen levels are generally inversely related to fish biomass (Piper et al. 1982). This inverse relationship led Cai and Summerfelt (1992) to hypothesize that oxygen consumption may be the limiting factor for increasing fish biomass within rearing tanks.

Results of this study indicate that metabolic rates vary for yellow perch at each loading density when exposed to a range of eugenol concentrations. In general, as eugenol concentration increased, metabolic rate of yellow perch decreased at each loading density tested. There were, however, no significant decreases in metabolic rates until yellow perch were exposed to 20 or 30 mg L⁻¹ eugenol. The dose-dependent oxygen consumption rate relationship I observed has also been documented with the sedatives 2-phenoxyethanol (Guo et al. 1995), isoeugenol (Forgan and Forster 2010) and clove oil (Hoskonen and Pirhonen 2004).

Eugenol exposure at 10 mg L⁻¹ did not significantly decrease metabolic rates relative to the control, conversely a higher metabolic rate was observed in eugenol exposed fish than the control at the 120 g L⁻¹ loading density. Few fish transport studies evaluated eugenol as the only active ingredient, therefore, the results of this study were compared with studies conducted using clove oil where eugenol is the primary active ingredient (70-90% by weight, Ross and Ross 2008). Results of several studies suggest that concentrations of less than 10 mg L⁻¹ can effectively reduce metabolic rate in fish (Cooke et al. 2004; Inoue et al. 2005; Iverson et al. 2009). For example, 5 - 8.5 mg L⁻¹ clove oil effectively reduced oxygen demand in largemouth bass *Micropterus salmoides* most likely because cardiovascular output was reduced (Cooke et al. 2004). Another study found that 5 mg L⁻¹ clove oil reduced the cortisol stress response of matrinxa *Brycon cephalus* when subjected to live transport, which should also decrease oxygen demand (Inoue et al. 2005). Finally, Iverson et al. (2009) measured the stress response of Atlantic salmon *Salmo salar* smolts exposed to clove oil during transport to sea and found that clove oil at 4.0 mg L⁻¹ was successful in reducing mortality. Results from this study at elevated loading densities suggest that a higher eugenol concentration (e.g. ≥ 20 mg L⁻¹ eugenol) is

needed to induce a reduction in metabolic rates for yellow perch. Additionally, to consistently significantly decrease metabolic rates, yellow perch should be exposed to 30 mg L⁻¹ eugenol for loading densities up to 240 g L⁻¹. The lower sedative concentrations from the previous studies can potentially be attributed to: (1) lower loading densities, (2) different physiological responses monitored, (3) temperature, (4) additional active constituents of clove oil and (5) different species tested.

The 22.2±2.7 g L⁻¹ loading density tested is much lower than typical transport density in production aquaculture (Carmichael et al. 2001). The significant reduction in metabolic rates when exposed to 30 mg L⁻¹ eugenol is important for aiding in successful transport for instances where maximizing the loading density during transport is not important (e.g. short duration transports <1 h, research purposes, and intra-hatchery operations). The similarity between reduced metabolic rates at the elevated loading densities and this light loading density also suggested that the eugenol concentration and not the loading density alter the metabolic rates of yellow perch when exposed to AQUI-S® 20E.

Tilapia

Guidelines for the transport of tilapia fingerlings suggest a maximum loading density of 120 g L⁻¹ (Timmons and Ebeling 2007). Due to their ability to survive in sub-optimal conditions and economic importance, I examined the effectiveness of eugenol to reduce metabolic rates at loading densities above those currently recommended.

I observed no significant differences in metabolic rates at the 240 and 360 g L⁻¹ loading densities when tilapia were exposed to eugenol. Deriggi et al. (2006) studied changes in plasma

composition of Nile tilapia when sedated in 50 mg L^{-1} eugenol for 10 min. No net ion imbalance was observed for juvenile tilapia, however, they observed a significantly longer time for plasma lactate levels to return to normal after exposure to eugenol. Although that study did not specifically monitor metabolic rates, they hypothesized that oxygen debt may have occurred during sedation because of a suppressed metabolic rate. By contrast, significantly lower metabolic rates at the 240 and 360 g L^{-1} loading densities were not observed in the present study. Therefore, eugenol concentrations up to 30 mg L^{-1} for 240 and 360 g L^{-1} loading densities may not induce the same physiological response as observed by Derrigi et al. (2006).

In another study, juvenile tilapia ($0.43 \pm 0.11 \text{ g}$) were placed into plastic bags at a loading density of approximately 22 g L^{-1} and exposed to 0, 9 and 18 mg L^{-1} clove oil for 24 h to assess changes in water quality and mortality that may occur during transport (Simoes et al. 2011). Significant effluxes in ions and increased mortality were observed for tilapia exposed to 18 mg L^{-1} clove oil. These results contradict those of Derrigi et al. (2006), but may be attributed to the use of much smaller tilapia and prolonged exposure to clove oil. The tilapia used were smaller those of the current study and those used by Derrigi et al. (2006), which may explain the lack of significant physiological changes observed at the medium and high loading densities due to sedation differing with fish size (Hoskonen and Pirhonen 2004).

Significantly lower metabolic rates were observed for tilapia exposed to 30 mg L^{-1} eugenol relative to tilapia exposed to no eugenol at the 120 g L^{-1} loading density, which represents a loading density that is commonly used in tilapia transport. Therefore, tilapia transported at this density may benefit from the use of 30 mg L^{-1} eugenol. Application of this

loading density and eugenol concentration combination is effective at reducing metabolic rates of tilapia, which is desired during live fish transport (Carmichael et al. 2001).

Tilapia exposed to eugenol at a loading density of $39.0 \pm 8.7 \text{ g L}^{-1}$ showed significantly lower metabolic rates when exposed to 30 mg L^{-1} eugenol relative to all other eugenol concentrations. Although no significant differences in metabolic rates were found at the three 240 and 360 g L^{-1} loading densities for tilapia exposed to 30 mg L^{-1} eugenol, the significantly lower metabolic rates found at the two fish and 120 g L^{-1} loading densities suggests that eugenol may be more effective at reducing metabolic rates at lower loading densities for tilapia.

CONCLUSION

When reduced metabolic rates of fish transported at higher than recommended loading densities are desired, much higher eugenol concentrations may be required than previously reported. Yellow perch exposed to 20 and 30 mg L⁻¹ eugenol (200 to 300 mg L⁻¹ AQUI-S®20E) at loading densities up to 240 g L⁻¹ had reduced metabolic rates. However, exposure to 30 mg L⁻¹ eugenol (300 mg L⁻¹ AQUI-S®20E) also led to variable metabolic rates in yellow perch. Tilapia exposed to 30 mg L⁻¹ eugenol (300 mg L⁻¹ AQUI-S®20E) at a loading density of 120 g L⁻¹ had reduced metabolic rates; AQUI-S®20E did not decrease metabolic rates at loading densities greater than 120 g L⁻¹. The results of this research suggest that exposure to AQUI-S®20E can lower metabolic rates of different species held at various loading densities. Exposure of yellow perch to AQUI-S®20E during transport may reduce metabolic rates at current or increased loading densities. Continued research is needed to characterize the response of tilapia to AQUI-S®20E during transport at high loading densities.

Future Research

There is a potential for oxygen debt to develop during prolonged exposure to AQUI-S®20E (e.g. during live transport). Additional research may be needed to address the potential for oxygen debt created by the use of eugenol during transport. Although this study showed that AQUI-S®20E is effective for decreasing metabolic rates during initial exposure, additional research is needed to examine the change in metabolic rates when fish are placed in sedative-free water post-transport. Additionally, further research should assess changes in metabolic rates at different durations of exposure to eugenol (i.e. metabolic rates at 2, 6 and 10 h of

exposure). Results from those types of studies may indicate the duration of effectiveness of eugenol during transport to decrease metabolic rates. Aside from monitoring oxygen metabolism, further exploration is needed to assess physiological changes in yellow perch and tilapia plasma constituents due to stress. Exposure to stressors has been shown to alter cortisol, lactate, glucose, electrolytes (i.e. Na^+ , K^+ and Cl^-), hemoglobin and osmolality of fish plasma (Ross and Ross 2008). Quantification of these specific responses to stressors would indicate the magnitude of physiological changes in fish during transport and provide an indication of the effectiveness of eugenol to mitigate those stress responses.

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Table 2.1. Mean (\pm SD) water quality parameters, total length (cm) and wet weight (g) during respirometry experiments. Temperature was measured and recorded while fish were in the respirometry chambers. pH was measured from stock solutions. Yellow perch had only one pH measurement recorded therefore no standard deviation was reported. Weight was measured prior to each trial and accounted for in loading density calculations.

	Total length (cm)	Wet weight (g)	Temperature (°C)	pH
Yellow Perch	10.1 (1.26)	9.74 (4.49)	16.2 (0.67)	7.93
Tilapia	11.4 (1.25)	25.91 (7.64)	21.5 (0.69)	7.95 (0.18)

Table 2.2. Loading densities (\pm SD) tested which were calculated from measured fish weights for yellow perch and tilapia.

	Loading Density (g L^{-1})			
Yellow Perch	22.2 (2.7)	59.5 (2.3)	121.9 (4.3)	243.4 (6.9)
Tilapia	39.0 (8.7)	116.2 (7.0)	215.6 (10.2)	309.6 (13.7)

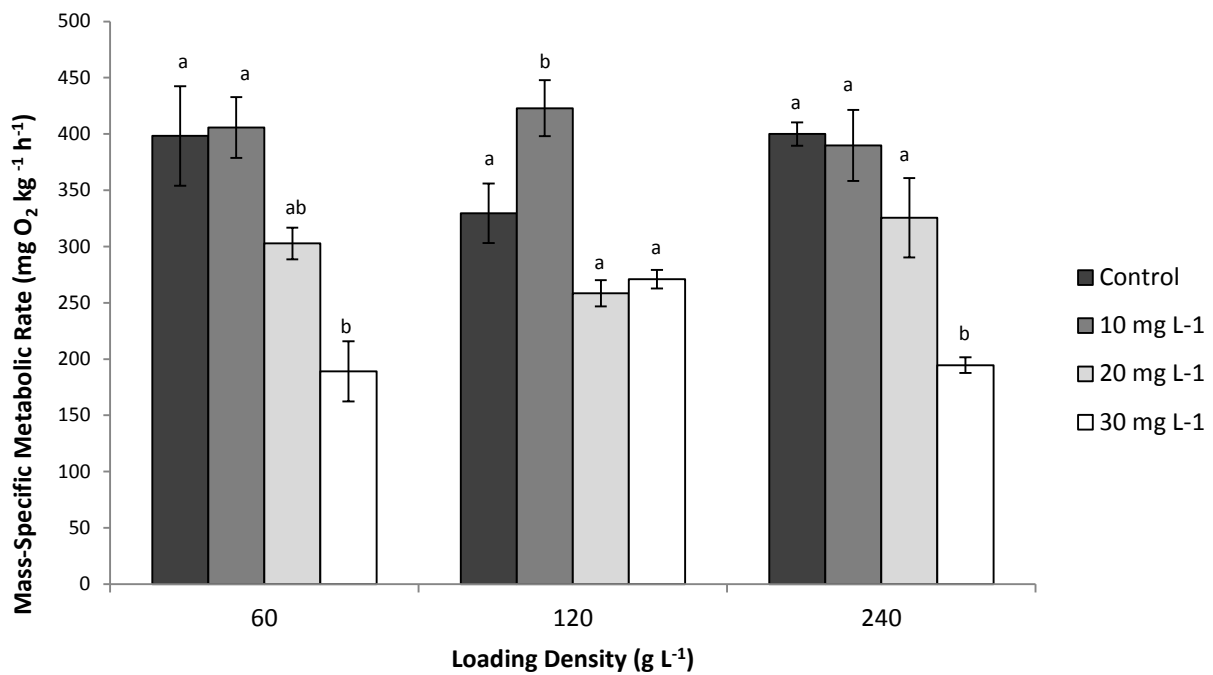
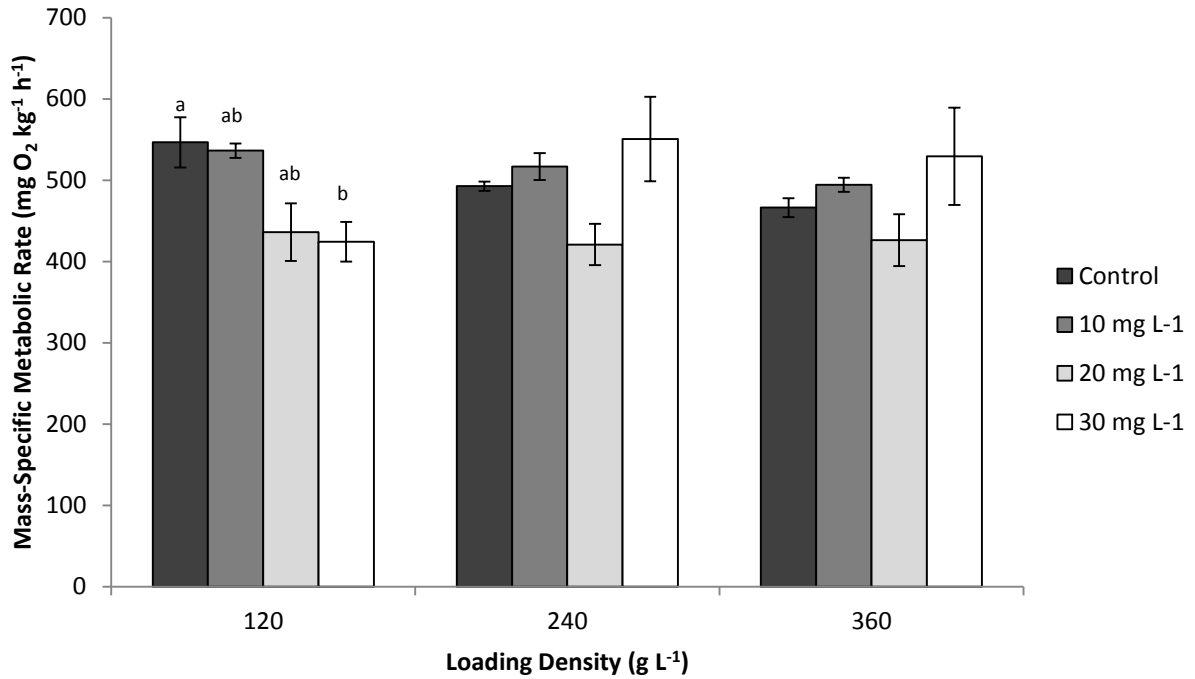


Figure 2.1. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia (top) and yellow perch (bottom) at three loading densities exposed to 0, 10, 20 and 30 mg L⁻¹ eugenol. Treatments with different letters are significantly different from each other ($p < 0.05$).

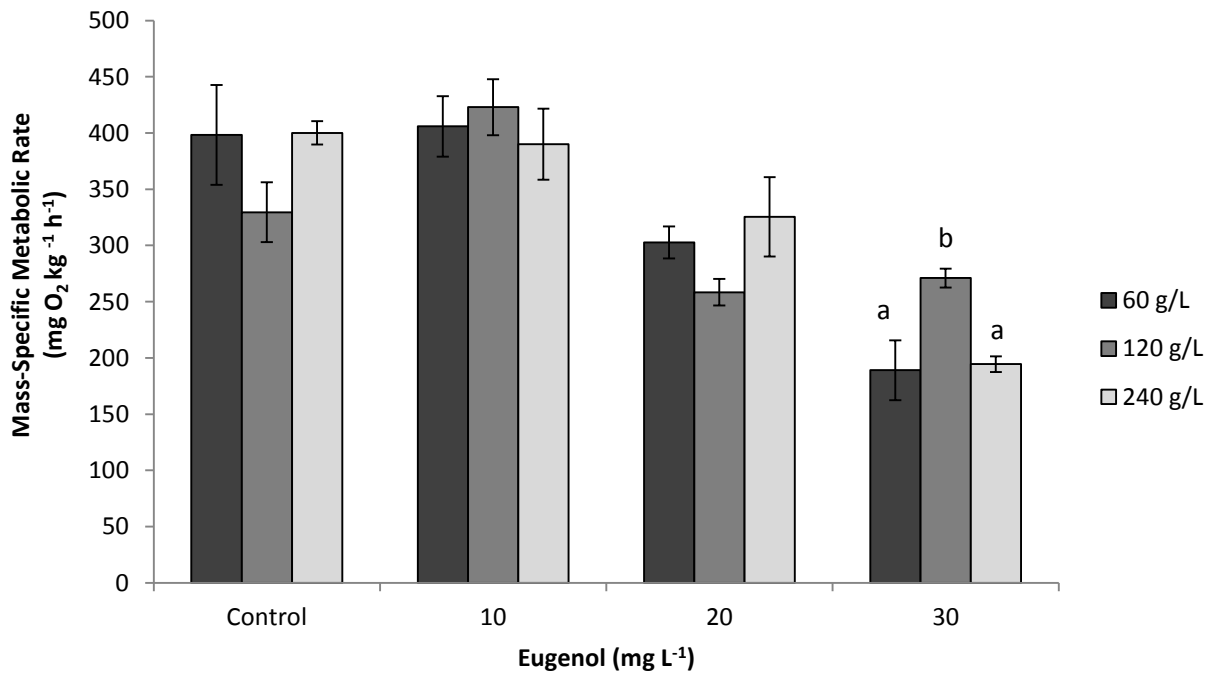
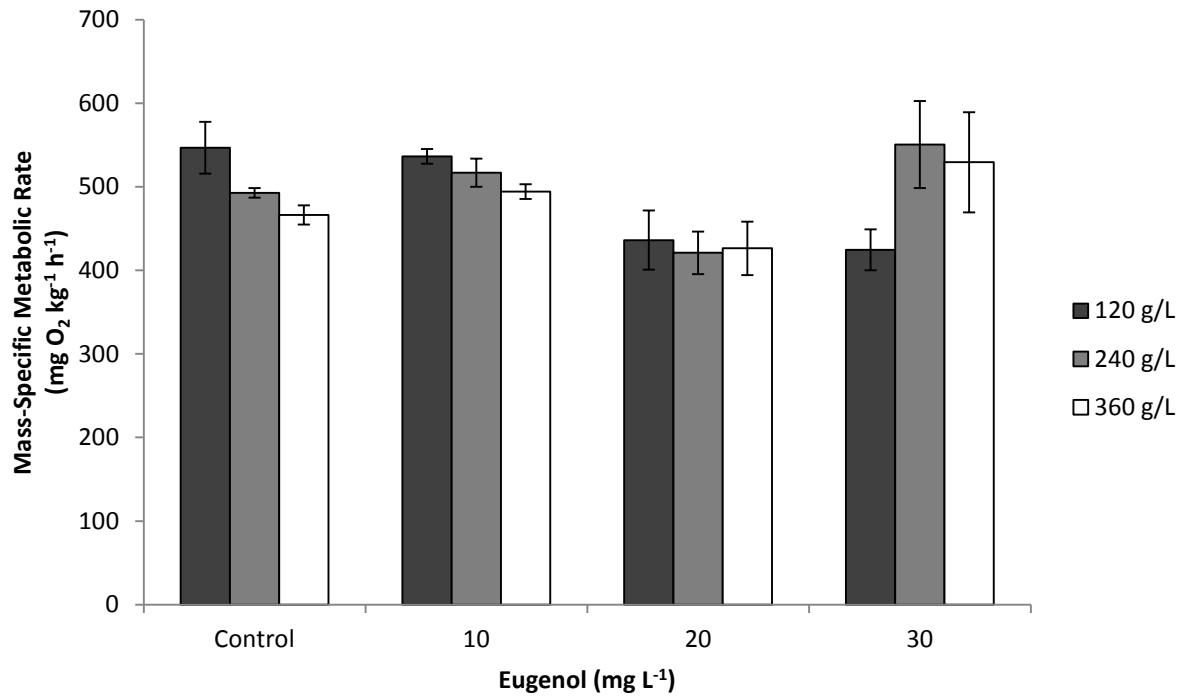


Figure 2.2. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia (top) and yellow perch (bottom) exposed to 0, 10, 20 and 30 mg L⁻¹ eugenol at three loading densities. Treatments with different letters are significantly different from each other ($p < 0.05$).

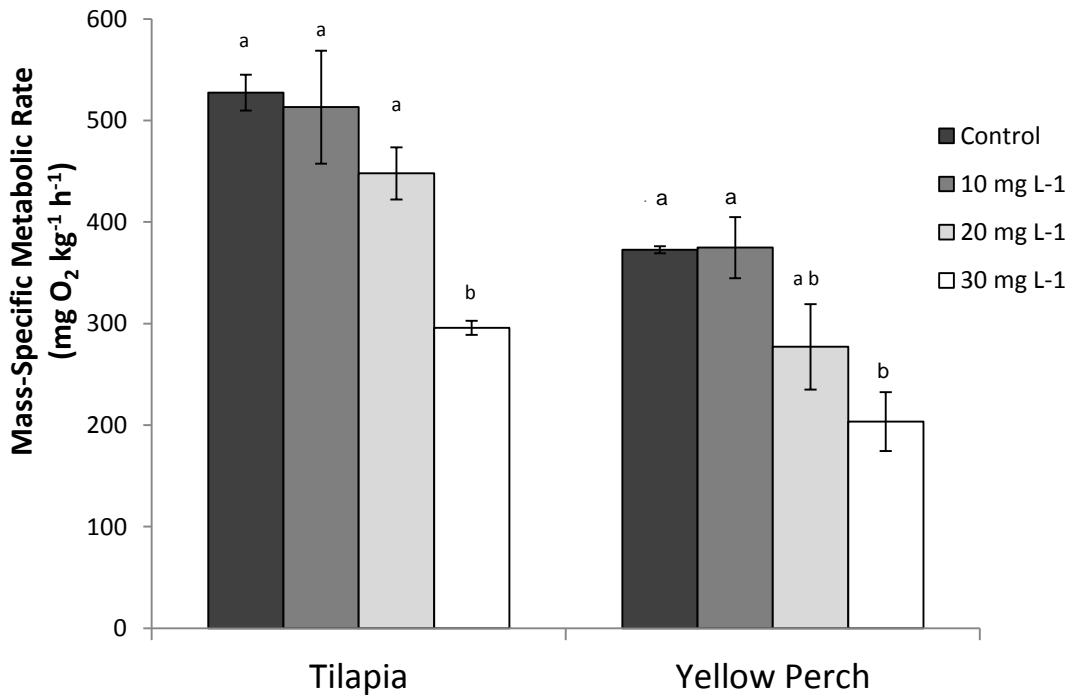


Figure 2.3. Mean mass-specific metabolic rates (\pm SEM bars) for tilapia and yellow perch at a loading density of two fish per trial exposed to 0, 10, 20 and 30 mg L⁻¹ eugenol. No interspecies rate comparison was analyzed. Treatments with different letters are significantly different from each other ($p < 0.05$).