LIVING WITH EMERGING CONTAMINANTS:
PROTEOMICS OF 4-NONYLPHENOL EXPOSED ARROW GOBY
(*CLEVELANDIA IOS*)

A Thesis
presented to
the Faculty of California Polytechnic State University,
San Luis Obispo

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Biological Sciences

by
Sarah Johnson
December 2011

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COMMITTEE MEMBERSHIP

TITLE: LIVING WITH EMERGING CONTAMINANTS:
PROTEOMICS OF 4-NONYLPHENOL EXPOSED
ARROW GOBY (*CLEVELANDIA IOS*)

AUTHOR: Sarah Johnson

DATE SUBMITTED: December 2011

COMMITTEE CHAIR: Dr. Lars Tomanek, Associate Professor

COMMITTEE MEMBER: Dr. Dean Wendt, Professor

COMMITTEE MEMBER: Dr. Emily Taylor, Associate Professor

ABSTRACT

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Sarah Johnson

Alkylphenol ethoxylates (APEs) are widely used in industrial and household products as surfactants. APEs degrade into more toxic ethoxylates, such as 4-nonylphenol (NP), which has been shown to be an endocrine disruptor and enhance the growth of tumor cells. Nonylphenol is wider spread in Pacific estuaries than originally thought. Organisms in Morro Bay, California contain some of the highest concentrations of NP reported, while containing few other contaminants. As a benthic mud-dwelling fish, the arrow goby (*Clevelandia ios*) may be exposed to high levels of NP due to high contaminant sequestration rates in anaerobic mud. While ecotoxicology suggests that nonylphenol is in high concentration within *C. ios* tissues along with tissue level biological abnormalities, the molecular effects of nonylphenol on these fish have yet to be investigated. Utilizing proteomic techniques including two-dimensional gel electrophoresis and subsequent identification via MALDI-TOF/TOF mass spectrometry, there is evidence for change in expression of proteins involved in energy metabolism, biotransformation, regulation and cellular structure.

Keywords: Proteomics, 4-nonylphenol (NP), *C. ios*

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my committee chairman, Dr. Lars Tomanek, for his guidance, support, and uncompromising attitude. I would like to thank my committee, Dr. Dean Wendt and Dr. Emily Taylor, for their passion and guidance, and recognition and celebration of all of my hard work.

Thanks to all members of the Environmental Proteomics Laboratory from 2006-2011; especially Betsey Turner, Megan Segal, Jennifer Diehl, and Loredana Serafini. Each of you has helped shape me as a scientist and an adult, for which I cannot express my gratitude enough.

Many thanks to all of the friends and graduate students who have kept me (relatively) sane throughout this whole process, including, but not limited to: Megan Segal, Loredana Serafini, Anniken Lydon, Sarah Knudsen, Johanna Weston, Melissa Daugherty, and James Koman.

A special thanks to my family: Liz, Paul and Rebecca, for encouraging me to follow my dreams, funding them, and then listening to me complain about them. Biggest thanks to my husband, Robert Bishop, for supporting me on both the good and bad days. I truly would not be who I am without my family and I thank you from the bottom of my heart for all of the opportunities you have given me and everything you have helped me to accomplish.

For the many gobies who gave their lives in the name of science.

I and this research have been funded by the Tenera Environmental Incorporated Scholarship (2009-2010); Archie and Andrea Waterbury Biological Sciences Scholarship (2009-2010); California State University Program for Education and Research in Biotechnology (2009 & 2007); California Sea Grant Graduate Student Traineeship (2008-2009); Doris A. Howell Foundation & CSUPERB (2008).

TABLE OF CONTENTS

LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
I. INTRODUCTION.....	- 1 -
Ecosystem.....	- 3 -
Morro Bay, California.....	- 4 -
The Arrow Goby (<i>Clevelandia ios</i>).....	- 7 -
Background.....	- 24 -
Chemical Analysis.....	- 26 -
Nonylphenol.....	- 30 -
Tools.....	- 53 -
Proteomics.....	- 54 -
Proteomics of Fishes.....	- 85 -
Ecotoxicoproteomics.....	- 89 -
II. MANUSCRIPT.....	- 98 -
Abstract.....	- 99 -
Introduction.....	- 100 -
Materials and Methods.....	- 103 -
Results.....	- 111 -
Discussion.....	- 128 -
Energy Metabolism.....	- 128 -
Biotransformation.....	- 130 -
Implications.....	- 144 -
REFERENCES.....	- 146 -

LIST OF TABLES

Table 1: Commonly used abbreviations.....	- 2 -
Table 2: Taxonomy of the arrow goby.....	- 10 -
Table 3: Sex Ratios of Female to Male Arrow Gobies, by California site	- 19 -
Table 4: Chemical structures of commonly used surfactants.	- 34 -
Table 5: Chemicals used in protein preparation for 2DGE	- 60 -
Table 6: Protein identifications (using MS/MS) of significant spots. Highlighted cells are interaction-significant, non highlighted cells are treatment-significant (p<0.01, 1000 permutations). Alphabetical order.	- 115 -
Table 7: Protein expression profiles of significant proteins involved in Energy Metabolism	- 119 -
Table 8: Protein expression profiles of significant proteins involved in Biotransformation	- 121 -
Table 9: Protein expression profiles of significant proteins involved in Amino Acid Metabolism	- 123 -
Table 10: Protein expression profiles of significant proteins involved in Structure	- 125 -
Table 11: Protein expression profiles of significant proteins involved in Regulation	- 127 -

LIST OF FIGURES

Figure 1: Morro Bay, Central California.....	- 4 -
Figure 2: Morro Bay Watershed, Marine Reserve, and Collection site used for this experiment (MBNEP)..	- 5 -
Figure 3: The arrow goby on the Morro Bay Mudflats. Photo credit Lars Tomanek.	- 7 -
Figure 4: Location of major <i>C. ios</i> investigations.....	- 9 -
Figure 5: Annual cycles of abundance, data from Prasad (1948), Macdonald (1972), and Brothers (1975).	- 15 -
Figure 6: Sexual dimorphism in arrow goby anal fins of A) females and B) males. Photo credit Sarah Johnson.	- 17 -
Figure 7: Female arrow goby with abdominal lesion, dissected open. Photo credit: Sarah Johnson	- 24 -
Figure 8: Percent macroscopic abdominal lesions observed since 2006, synthesized by season. Average percent seen in catch is 5.7%; spring n=354, summer n=336, fall n=78, winter n=347.	- 24 -
Figure 9: From Bevan et al., 2003: "Figure 2.Representative examples of (A) an embryo maintained in vehicle alone (EtOH) and (B) one maintained in saline containing 1 μ M nonylphenol beginning at stage 10.5 for 48 hr (~stage 37 for control embryos). Crooked backs, kinked tails, poorly developed somites and dorsal fins, and "chicken-beaked" cement glands (CG) were often evident in embryos treated with micromolar concentrations of the estrogenic compounds, E2, octylphenol, or nonylphenol.	- 49 -
Figure 10: Two-Dimensional Gel. 100ug arrow goby liver protein. 4-7pH IPG strips, 11% PAGE, SYPRO ruby stain.	- 54 -

Figure 11: 2DGE workflow, A) Experiment, B) Protein preparation including homogenization, precipitation and quantification, and protein separation including C) First dimension isoelectric focusing (biorad) and D) Second dimension SDS-PAGE (biorad).	- 58 -
Figure 12: Isoelectric focusing.	- 65 -
Figure 13: Protein quantification with gel image analysis software, including A) image warping, B) image fusion, C) spot detecting and editing, D) application of consensus spot pattern, and E) extracting expression profiles (Berth et al., 2007).	- 69 -
Figure 14: Mass Spectrometer A) Environmental Proteomics Laboratory MALDI-TOF/TOF, B) MALDI-TOF/TOF and LIFT diagram (Suckau et al. 2003).	- 75 -
Figure 15: PMF and MS/MS (inset) from mass spectrometer, protein identified as ATP Synthase F1 β -subunit [Mowse score = 292]	- 77 -
Figure 16: Concentration of nonylphenol found in whole animals of <i>C. ios</i> (GI tract removed) due to laboratory exposure. N=3 for each point, standardized for lipid concentration.	- 111 -
Figure 17: 2DGE fused image results. 100 μ g <i>C. ios</i> liver tissue, 4-7 pH, stained with SYPRO ruby.	- 113 -
Figure 18: Heat map of all significant and identified proteins. Columns are individual fish, broken into groups: C = control, NP = nonylphenol exposed. Rows are individual proteins. Blue represents down-regulation, red is up-regulation. Figure includes interaction and treatment proteins separated, and organized through hierarchical clustering.	- 117 -
Figure 19: Catabolic pathways for phenylalanine and tyrosine. (Fig 18.23 from Lehninger et al., 2004).	- 135 -

Figure 20: Summary of amino acid catabolism (Fig 18.15 from Lehninger et al., 2004)	- 137 -
Figure 21: Summary of cellular effects of nonylphenol. Blue represents down-regulation, red is up-regulation, green is other. Pink rectangles are amino acids undergoing catabolism.	- 142 -
Figure 22: Summary of the effects of nonylphenol....	- 143 -

I. INTRODUCTION

Anthropogenic pollution is a worldwide phenomenon and threat to the well-being of many organisms, including our own species. This thesis concentrates on the cellular response of the arrow goby to exposure to the contaminant 4-nonylphenol. The distribution of NP within our own Morro Bay estuary, as well as within estuaries along the west coast is discussed in the paper: Ubiquitous distribution of nonylphenol in west coast estuaries (Diehl, Johnson, Xia, West, and Tomanek, *in preparation*).

This chapter is broken into three sections, the first dealing with the ecosystem found in Morro Bay, California, and the arrow goby. Next, the background and implications are weighed through the histopathology and chemical analysis of the fish, along with a review of current and relevant nonylphenol research. Finally, the appropriate tools for an investigation of this magnitude are investigated, including proteomics and ecotoxicoproteomics. A list of commonly used abbreviations is included (Table 1).

Table 1: Commonly used abbreviations

Abbr.	Explanation
1D	First dimension isoelectric focusing
2D	Second dimension SDS-PAGE
2DGE	Two-dimensional gel electrophoresis
APE	Alkylphenol ethoxylates
MALDI-TOF/TOF	Matrix-assisted laser desorption/ ionization time-of-flight/time-of-flight, a type of mass spectrometer
MBPP	Morro Bay Power Plant
MoA	Mode of action, referring to chemical toxicity
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry, a.k.a. LIFT
MSCL	Mississippi State Chemical Lab
NP	Nonylphenol, 4-Nonylphenol
NPE	Nonylphenol ethoxylates
PES	Protein expression signatures
pI	Isoelectric focusing point
PMF	Peptide mass fingerprint
PTM	Post translational modifications
ROS	Reactive oxygen species
SDS-PAGE	Sodium-dodecyl-sulfate poly acrylamide gel for electrophoresis
SLOSEA	The San Luis Obispo Science and Ecosystem Alliance
WQC	Water quality criteria
WW	Wet weight
WWTP	Waste water treatment plant

Ecosystem

In this section the study site of Morro Bay, California is discussed, along with the study organism: the arrow goby (*Clevelandia ios*).

Morro Bay, California

Figure 1: Morro Bay, Central California.

Morro Bay is located on the Central Coast of California, in San Luis Obispo County (Figure 1). The town was founded by Franklin Riley in 1870, "because he had a vision of the future" (Castle and Ream, 2010). Today the city of Morro Bay is a popular tourist destination, while the bay is best known as a bird sanctuary, a state and national estuary, as well as a marine reserve. There is a low human population density of about 128 people/ km², surveyed in 2000 (Diehl et al., 2011).

Bays and estuaries are coastal environments that are partially protected by land, making them a refuge from the full force of the ocean (Barton, 2007). Estuaries are the result of fresh water draining into bays, so that the water

is brackish, i.e. has a sodium concentration between that of marine and freshwater habitats. As this concentration fluctuates diurnally, tidally and seasonally, most estuarine organisms are euryhaline osmoregulators (Barton, 2007).

Figure 2: Morro Bay Watershed, Marine Reserve, and Collection site used for this experiment (MBNEP).

Rivers bring in freshwater, sediment, and organic and inorganic nutrients. The two main freshwater inputs to Morro Bay are Chorro and Los Osos Creeks (Figure 2). These creeks receive inputs from farmland and cities before

entering the bay, and are therefore potential sources of runoff into the bay. The total Morro Bay watershed covers 19,425 hectares (Tenera, 2001).

Morro Bay is considered a shallow estuary and is 6.9 km long by 2.9 km wide (Tenera, 2001). At high tide there are 850 hectares of surface water in the bay, while at low tide there are 528 hectares of mud flat exposed (Tenera, 2001). In this fluctuating environment, estuarine organisms must often tolerate a wide range of environmental factors, and have developed useful adaptations such as a wide instead of a narrow tolerance range to temperatures (eury- versus stenothermal) (Barton, 2007). For the purposes of this project, *Cleavelandia ios* were collected on the mudflats, south of the Morro Bay Marina (+35° 20' 41.28", - 120° 50' 39.54").

The Arrow Goby (*Clevelandia ios*)

**Figure 3: The arrow goby on the Morro Bay Mudflats.
Photo credit Lars Tomanek.**

The arrow goby (*Clevelandia ios*) is a small benthic fish that can be found in estuaries, lagoons and tidal sloughs along the west coast of North America, from Baja California to British Columbia (Figure 3; Eschmeyer et al., 1983). *C. ios* is commensal with the ghost shrimp (*Neotrypaea californiensis*) the fat innkeeper worm (*Urechis caupo*), and the blue mud shrimp (*Upogebia pugettensis*; Macdonald, 1972). The arrow goby prefers muddy, silty substrates over sand and is easily located in invertebrate holes at low tide with yabbie pumps (a.k.a shrimp guns, or slurp guns) on the mudflats, or at high tide with underwater seine nets. During low tides *C. ios* may choose to remain on the exposed mudflats (Figure 3), where it is

likely that the fish respire through its integument; or *C. ios* can use the invertebrate burrows as shelter, while the residents are unaffected by the presence of the fish. As a benthic, estuarine fish, the arrow goby is subjected to daily fluctuations in tide, salinity, and temperature; as well as seasonal runoff. Considering the environmental conditions of *C. ios*, it is not surprising that it is thought to have a wide tolerance range against a number of environmental stresses.

With no commercial interest in the species, information on *C. ios* is dated and limited. Major investigations include three Ph.D. theses, molecular work in conjunction with the tidewater goby (*Eucyclogobius newberryi*) and abundance data relating to power plant cooling issues.

Major life history investigations of *C. ios* have been undertaken by Prasad (1948), Macdonald (1972) and Brothers (1975). All three of these include data on abundance, behavior, taxonomy and comparative morphology. Each of the investigators concentrated on a local population of *C. ios* in Elkhorn Slough (Monterey County, Ca), Anaheim Bay (Orange County, Ca), and Mission Bay San Diego (San Diego County, Ca), respectively (Figure 4).

Figure 4: Location of major *C. ios* investigations.

Taxonomy and Genetics

Clevelandia ios was first described in 1882 by David S. Jordan and Charles H. Gilbert. These professors found a female specimen in 1880 in the stomach of a greenling (*Hexagrammus asper*) on Vancouver Island, British Columbia, Canada. They found that the “specimen has been somewhat injured by the process of digestion” and designated it *Gobiosoma ios* (Jordan and Gilbert, 1882). In Greek, the

word “ios” means arrow, which is the characteristic shape of the arrow goby when viewed from above.

More than half of all living vertebrate species are fishes (Barton, 2007). Gobies are in the class Actinopterygii for their rayed-fins, c.f. sarcopterygian lobe-finned fishes (Table 2). The arrow goby is in the order Perciformes for being perch-like and inhabiting shallow-water marine habitats. *C.*

ios resides in the family Gobiidae

which is the largest family in the suborder Gobioidae (Barton, 2007).

There are about 212 genera and nearly 1,900 species in this family, making the goby family the largest family of marine fishes. Members of the Gobiidae family are small, often less than ten centimeters long, and have fused pectoral fins (Barton, 2007).

The arrow goby is currently the single species in the genus *Clevelandia*, however Jordan and Gilbert (1882) originally placed

Taxonomy of arrow goby.

the arrow goby in the genus *Gobiosoma* because they thought the fish lacked scales. Actually, juvenile arrow gobies lack scales, which develop after the fish has reached a standard length of 18.5mm (Prasad, 1948). However, in 1895 it was discovered that the fish does indeed possess scales, influencing Jordan and Starks to move the arrow goby to the genus *Clevelandia*, named for Daniel Cleveland of the San Diego Society of Natural History (Macdonald 1972). This genus was originally characterized by four dorsal spines and weak minute cycloid scales. As these characteristics are rather general, it was suggested that the arrow goby be placed in the genus *Gillichthys*. Eigenmann reexamined *Clevelandia* in 1891 and found its skull to be smooth while *Gillichthys* was depressed, therefore rejected the idea of lumping these two groups together (Macdonald, 1972).

Briefly, the genus included *Clevelandia rosae* and *Clevelandia longipinnis* described by Jordan in 1896 (Macdonald 1972). However, these turned out to be the same fish, which was ultimately designated *C. rosae*. In 1938, Ginsburg suggested that all genera of gobies closely related to *Lepidogobius* be placed in that genus. However, "the scientific name *Clevelandia ios* is so thoroughly entrenched in the literature that any further generic change would prove difficult," thus the name was retained

and the arrow goby remains the only fish in the genus *Clevelandia* (Macdonald, 1972).

What little genetic investigation has been completed with *C. ios* is in concert with its endangered sympatric sister species, the tidewater goby (*E. newberryi*). Sympatrics occur in the same geographical region, and sister species imply that they are the most closely related to each other (Gotelli, 2008). Based on genetic data on variation in the mitochondrial control region (mtCR), it seems that there is high gene flow, and low genetic diversity in arrow goby populations along the west coast of North America (Dawson et al., 2002).

Dawson looked at samples from Northern and Southern California, and found no evidence of isolation by distance. Therefore, it is likely that there is a large amount of gene flow between *C. ios* populations along the coast of California. This supports the notion that populations of arrow goby may actually be panmictic (Gotelli, 2008).

Greater dispersal ability can result in greater gene flow, together contributing to a more "shallow" genetic population structure (Dawson et al., 2002; Gotelli, 2008). These greater dispersal abilities often result from interaction of habitat and life history (Dawson et al., 2002). Dawson explored the fact that geographically co-

occurring sympatric sister species (e.g. the arrow and tidewater goby) share the most recent common phylogenetic history and therefore differences in their phylogeography should be attributed to recently derived, species-specific characteristics (Dawson et al., 2002).

Dawson concludes that abiotic factors influenced cladogenesis between *C. ios* and *E. newberryi*. Arrow gobies reside in tidal habitats, open year round to oceans; while the tidewater goby can be found in habitats that are not only non-tidal, but are closed to the ocean during times of reproduction. The difference in accessibility to the ocean may be a reason why *C. ios* has greater dispersal rates than *E. newberryi*, which lead to a shallower phylogeographic structure.

Previous to genetic work, Prasad (1958) explored the idea of different "races" of *C. ios*, based on meristic and morphometric characteristics. He looked at fin ray counts, and body measurement differences in four California populations of *C. ios*: Elkhorn Slough, Mugu Lagoon, Mission Bay and Tijuana Slough (Prasad, 1958). He found that each population differed from one another in several measurements: first dorsal fin-rays, second dorsal fin-rays, anal fin rays, and length of head. Prasad found that the northern Elkhorn Slough population differed from the

southern Mission Bay and Tijuana Slough populations in all characters. He concluded that populations that are further apart geographically show greater differences in characteristics than those that are closer to each other (Prasad, 1958).

Based on these differences between populations, Prasad called each population of *C. ios* a different race (Prasad, 1958). Prasad assumes that "the populations are restricted to definite areas and the chances of these mixing are rather few," indicating he believes genetic isolation is important component in determining differences between populations.

From Dawson's genetic work, we know that there is quite a bit of gene flow between populations. Given the fact that there is genetic homogeneity, differences in phenotype are not DNA based, but likely environmentally determined. So in this case, race is not a genetic difference between populations, but a phenotypic difference due to environmental control. Given the same genotype, different phenotypes can be expressed under different conditions. Fish phenotypic expression can be plastic, e.g. water temperature during development can influence the number of vertebra and fin rays (Barton, 2007; Gotelli, 2008). Furthermore, different phenotypic characteristics

can be referenced to a given population, i.e. emergent traits (Gotelli, 2008). So rather than Prasad's postulated genetically distinct races, there seems to be phenotypically distinct populations of *C. ios*, due to weak environmental selection, i.e. selection that is not strong enough to cause speciation.

Life

Much of the life history of *C. ios* has been explored by Prasad in Elkhorn Slough (1948), Macdonald in Anaheim Bay (1972) and Brothers in Mission Bay San Diego (1975). Synthesizing the extensive abundance data from all three of theses yields a similar distribution (Figure 5).

Figure 5: Annual cycles of abundance, data from Prasad (1948), Macdonald (1972), and Brothers (1975).

The three investigators saw a fluctuation in population size over a one-year period (Prasad, 1948; Macdonald, 1972; Brothers, 1975). They all saw an increase in population size in the late summer, which corresponds to

juveniles hatched in the spring. However, the three investigators used slightly different collection methods (i.e. slurp-gun and/or seine net) and had different catch sizes so these data may not be 100% comparable.

The arrow goby lifespan is roughly two years: Prasad found that most of a *C. ios* brood is completely gone two years after its initial peak spawning period (Prasad, 1948); and Macdonald (1972) investigated arrow goby otoliths (ear bones, commonly used to age fish [Barton, 2007]), found that most fish live between one and two years.

Because ovarian development is non-synchronous, females can be found at various stages of oogenesis, which allows the mating season of *C. ios* to be almost year-round, though spikes are seen in the spring (Tenera, 2001; Prasad, 1948; Macdonald, 1972). Arrow gobies are oviparous, laying demersal eggs that are negatively buoyant and sink (Tenera, 2001).

The female can lay up to one thousand eggs at a time, in small groups of fifteen to twenty-five. Arrow gobies do not build nests or have specialized spawning areas, c.f. other fish in the Gobiidae Family (Prasad, 1948). There is no parental care exhibited by *C. ios*, and the fish "seems

quite unconcerned about the eggs," which are deposited over a large area and not in nests or burrows (Prasad, 1948).

Prasad (1948) explored the length of *C. ios* at various stages of maturity from Elkhorn Slough. Maturing females are those that contain developing eggs, while immature fish are those that have never spawned (Prasad, 1948). He found all fish less than 29 mm to be immature, and therefore termed 29 mm the "size at first maturity". With each millimeter of growth, he found an increase in the percent of maturing females, and after fish had reached a length of 34mm, 100% of them were mature and contained spawnable eggs (Prasad, 1948).

Figure 6: Sexual dimorphism in arrow goby anal fins of A) females and B) males. Photo credit Sarah Johnson.

As *C. ios* matures, there is sexual dimorphism between male and female fish (Figure 6). Generally, there is a black band through the midportion of the anal fin on male arrow gobies, while females have transparent anal fins (Eschmeyer et al., 1983). There are slight color changes in both males and females during the breeding season, which

persist several days before spawning and disappear after spawning. For females there is an increase in melanophore pigmentation on both dorsal fins and in pigmentation all over the body, as well as a bit of dark color on the anal fin at this time (Prasad, 1948). The female's abdomen becomes fully distended because she is gravid. Her abdomen is often a yellow shade, due to the eggs shining through the skin (Prasad, 1948). This often leads to sluggish behavior, due to the extra bulk she is carrying around.

In contrast, the males remain very active and do not have any changes in abdomen size. Before spawning the characteristic black band on the anal fin becomes very dark, and will fade after discharge of sperm. Males will also have an increase in melanophore pigmentation on the dorsal fins, on the tip of the pectoral fins, and on the lower jaw (Prasad, 1948).

Like most fish, *C. ios* exhibits a type III survivorship curve or life strategy. These organisms tend to spend their energy on a large number of offspring (high fecundity) rather than in long-term care of offspring, which is a type I strategy. There is often early reproduction and low survivorship of offspring, but sheer numbers can maximize reproductive success (Gotelli, 2007).

The arrow goby's mating system is a reflection of its life strategy.

Table 3: Sex Ratios of Female to Male Arrow Gobies, by California site

Population	Sex Ratio (F/M)	Difference in Body size	N=
Elkhorn (Prasad, 1948)	2/1	ND	1269
Anaheim (Macdonald, 1972)	3.3/1	ND *head size	713
San Diego (Brothers, 1975)	1.1/1	Females > males in same age group ($p < .05$)	4488
Morro Bay (Johnson, 2010)	2.3/1	Not measured	1306

In monogamous species, there is usually a one-to-one ratio of males to females. Any deviation from this will lead to reproductive variance and differential reproductive success for those involved. That being said, it is possible that there is a larger proportion of female to male arrow gobies (Table 3). Prasad (1948) estimated there to be twice as many females as males in the Elkhorn Slough population, while Macdonald estimated over a three-to-one female biased population in the Anaheim population (Macdonald, 1972). However, with changes in collecting techniques, Brothers suggests that the former sex biases may actually be collection biases. He found only a slightly higher number of females in his San Diego population. Brothers suggests that Prasad and Macdonald's

use of seine nets is a sex biased collection method that selects for the slower moving, gravid females more readily than males. Brothers also used seine nets, but included slurp guns for a more "unbiased" approach. Brothers even points out that in one of his collections regarding only fish collected via seine net, that he found a two-to-one female sex bias (Brothers, 1972). However, I have always noticed a heavy female sex bias in the Morro Bay population, sampling exclusively via yabbie pump (Table 3). Further research needs to be done to elucidate how much of the sex ratio is an artifact of collection, a population level variation or even potential endocrine disruption (see page 36). If there truly is a female bias in the arrow goby populations, it is unclear if this is due to initial sex ratios of eggs, or if this is due to differential survivorship between the sexes.

Another trait that can be indicative of life history is differential body sizes between males and females. Prasad and Macdonald did not find any significant difference in body length between the sexes (Table 3). However, Macdonald did find a significant difference in adult head length, with males having a longer head than females (Macdonald, 1972). Again, Brothers has new insight into his predecessors' data: he compared males and females

of the same age category, and found that females are larger than males of the same age group (Brothers, 1975). I have also noticed that females in Morro Bay on average tend to be larger than males (data not shown). In monogamous species, males and females tend to have similar body sizes and coloration. Because there is sexual dimorphism we can infer that the arrow goby is not monogamous. Biased sex ratios and differential body sizes are characteristic of an organism that is not monogamous (Gotelli, 2007). Furthermore, monogamy is often marked by parental care, and *C. ios* is a broadcast spawner.

Behavior

Again, *C. ios* is commensal with the ghost and blue mud shrimps and the fat innkeeper worm (Macdonald, 1972). A subset of symbiosis, this is a commensal relationship as the arrow goby benefits by using the shrimp's burrow for protection, and the shrimp is neither harmed nor benefits from the presence of the arrow goby. The fish uses the burrow for protection, and potential food acquisition,

if a large piece of, for example, clam meat, too large for a goby to swallow, is put into a burrow which also contains crabs the goby, after attempting to swallow or tear it apart, will carry the meat to a crab and stand by while the latter makes it smaller, the fish at intervals snatching it for another attempt at swallowing, to the disconcertion of the crab. (MacGinitie, 1934)

The arrow goby's intraspecific behavior has been called "agnostic" (c.f. antagonistic) because of its lack of aggression, e.g. site-specific defense or intraspecific dominance. Arrow gobies can be found in close proximity to conspecifics, and often several fish can be found in the same burrow at once (Brothers, 1975). Furthermore, moving with the tides makes it nearly impossible to have territorial defense.

In 1972 Macdonald made several observations about *C. ios* behavior both in the field and from twenty male and twenty female fish in an aquarium. First, Macdonald only noticed fighting behavior once during a feeding session, likely related to social hierarchy. There was some chasing and nipping done by the largest male, which displayed typical threat behavior by expanding both the anal and dorsal fins, and a fully opened mouth. In my own studies with *C. ios*, there is evidence for a social hierarchy because the largest fish in a tank will feed before the smaller ones, though I did not observe any chasing or nipping during feeding (data not shown).

C. ios exhibits several signaling behaviors utilizing its guanine lined peritoneum. This gives the abdominal region of the adult goby a characteristic silvery color that can be used to signal others in the case of danger

(Macdonald, 1972). During high tide the arrow goby can be found swimming near the shrimp burrows. When danger threatens, a fish will flip upside down and dive head first into a burrow. Light will flash from the abdominal region, warning others nearby to do the same. This will cause a "chain reaction" and within a few seconds the entire population of a mudflat can be found in the invertebrate burrows (Macdonald, 1972). This sentinel behavior is a type of kinship selection, and is often seen in organisms that are related to one another (Hamilton, 1964). Male arrow gobies also take advantage of this as a mating behavior. During twilight, the male will stop swimming. He will use his tail to lunge forward, arc off the bottom and roll to his side. This can be repeated as many as ten times (Macdonald, 1972).

Background

Figure 7: Female arrow goby with abdominal lesion, dissected open. Photo credit: Sarah Johnson

Beginning with the first collection of *C. ios* in the summer of 2006, we noticed strange growths on some of the fish collected from Morro Bay (Figure 7). To assess frequency, we collected a greater number of fish and found macroscopic growths in 5.8% of our catch (Figure 8).

Figure 8: Percent macroscopic abdominal lesions observed since 2006, synthesized by season. Average percent seen in catch is 5.7%; spring n=354, summer n=336, fall n=78, winter n=347.

An initial histopathological analysis yielded several tissue level abnormalities including liver and gonadal

tumors (Dr. Swee Teh, personal communication). In contrast, a more recent analysis has yielded the possibility that these growths are abdominal lesions, potentially caused by a microsporidia parasite (Dr. Mark Myers, personal communication).

Hoping to pin-point a causal agent, a chemical analysis was performed. We tested *C. ios* livers for over 100 chemicals, including PCBs, PBDEs, PAHs, hormones, legacy and current-use pesticides, butyltins, heavy metals, and industrial and personal use chemicals. The chemical in highest concentration in the fish tissues was a compound called 4-nonylphenol (NP), which would become the center of our work.

There are ongoing efforts by the Environmental Proteomics Lab to investigate these growths, and future research projects may elucidate their cause and pathology. While these growths were the catalyst for this project, the fact remains that the Morro Bay arrow goby population has extremely high levels of nonylphenol in their tissues and the focus of this thesis is on the physiological effect of nonylphenol exposure on the arrow goby.

Chemical Analysis

Using gas chromatography-mass spectrometry (GCMS), the chemical components of a tissue or solution can be analyzed. Our chemical analysis was performed by Dr. Kang Xia at the Mississippi State Chemical Lab, MS.

We analyzed whole fish samples of *C. ios* (minus digestive tracts, due to potential contamination of ingested sediment) collected in April and July 2009 for over 122 analytes, including PCBs, PBDEs, PAHs, hormones, legacy and current-use pesticides, butyltins, heavy metals, and industrial and personal use chemicals, and we analyzed two samples of *C. ios* livers for over 75 of these same analytes from fish collected in January 2008 and April 2009. In order to meet the minimum tissue requirements for analyses of analytes in goby livers, 230 livers were pooled for the January 2008 sample and 209 livers were pooled in the April 2008 sample. The livers in the January 2008 sample were obtained from 65 male and 165 female gobies, 15 of which had macroscopic gonadal lesions; the April 2009 sample was comprised of livers from 52 male and 157 female gobies, 18 of which had macroscopic gonadal lesions. Whole fish samples contained between 2 and 10 gobies for 4-nonylphenol analyses. Whole fish samples for butyltin species analysis required 52 fish (23 male and 29 female, 1

with a macroscopic abdominal lesion). A combined analysis for heavy metals, BPA, BHA, BHT, and bis (2-ethylhexyl) phthalate required 80 fish (30 male and 50 female, 2 with visible abdominal lesions). Analysis for PAHs in whole fish tissue required 35 fish (5 male and 30 female, 2 with macroscopic abdominal lesions). Regression analysis showed no relationship between number of fish in a sample and the concentration of NP detected ($y = 145 + 2.4x$; $r^2 = 0.001$, $f_{1,25} = 0.03$, $p = 0.875$); similarly, early samples segregating males and female fish for NP analysis failed to show any gender bias in concentration of NP (t-test: $t_{13} = -0.01$, $p = 0.99$), so subsequent samples combined genders indiscriminately (Diehl et al., 2011).

Most of these compounds came back under the detection limit, which immediately ruled them out as potential causative agents of the *C. ios* lesions. *C. ios* livers contained only four analytes: 4-nonylphenol, DDT metabolites, some PCBs, and trans-nonachlor. 4-NP levels in the goby livers were the highest of the contaminants tested: 716 ng/g and 2700 ng/g wet weight (ww), from the January 2008 and April 2009 samples, respectively. Whole fish tissue also contained 4-nonylphenol and DDT metabolites, though at lower concentrations than in lipidic

liver tissue; however, PCBs and trans-nonachlor were not detected (Diehl et al., 2011).

Butyltin species, metals, and PAHs were only analyzed in whole fish samples, and we found that goby tissue contains the four butyltin species, many metals (Al, Fe, Hg, Mn, As, Cr, Cu, Ni, Se, Zn), diazepam, triclosan, and only one PAH (phenanthrene), present at the detection limit of 10 ng/g. Tributyltin and its degradation compounds, dibutyltin and monobutyltin, are introduced into marine environments from anti-fouling paints and are a concern for marine life due to their inhibition of growth and reproduction in marine invertebrates (Santillo et al., 2001). Wet-weight concentrations of monobutyltin and dibutyltin in whole tissue of *C. ios* were comparable to or lower than levels reported in muscle of marine fishes of previous studies, but levels of tributyltin in *C. ios* were considerably lower than levels in other marine fishes (Harino et al., 2000; Senthilkumar et al., 1999). The relatively low levels of the parent compound, tributyltin, to its degradation products in Morro Bay indicate that inputs of tributyltin to the bay have decreased or ceased in recent years (Diehl et al., 2011).

Many of the heavy metals present in *C. ios* tissue were at concentrations greater than other analytes tested.

Toxicity levels of heavy metals can be assessed with the Effects Range Low (ERL) values for marine sediments where each ERL represents the concentration below which less than 10% of studies found an adverse biological effect of the metal (Long et al. 1995, Long et al. 1998). Since ERL levels are dry weight values, we used the average percent moisture from all whole goby samples (81.4%) to calculate the dry weight values of heavy metals in goby tissue. Only zinc and mercury exceeded ERL values (Diehl et al., 2011).

Interestingly, the only chemical to exceed its ERL guideline, 2.2 ng/g (dw), was a breakdown product of the well-known pesticide DDT; p,p'-DDE was the second most concentrated chemical in whole fish and liver tissue despite the cessation of agricultural application of DDT to the environment in 1972. DDT is a synthetic chemical similar to NP in that they are both hydrophobic, adhere to organic matter and soil, persist for decades, and act as endocrine disruptors due to their xenoestrogenic properties. 4-NP is not currently a chemical of concern, so ERL guidelines are not available (Diehl et al., 2011).

Nonylphenol

The effect of nonylphenol on organisms has been the subject of numerous studies and what follows can only constitute a brief summary of findings.

Background, History, Politics

Nonylphenol (NP) is not a naturally occurring compound, and was first synthesized in 1940 as a by-product during oil-refinement, and has been found to be useful as a building block for surfactants (Soares et al., 2008). The primary source for NP in the environment is the breakdown of alkylphenol ethoxylates (APEs) which are a widely used class of surfactants (Ying et al., 2002).

APEs are one of the most widely used surfactants, and are used as detergents, wetting agents, dispersants, emulsifiers, solubilizers and foaming agents (Ying et al., 2002). Fifty-five percent of APEs are used in industry, i.e. paper, textiles, coatings, pesticides, lube oils and fuels, metals and plastics (Ying et al., 2002). The remainder of the APE market includes 30% industrial and institutional cleaning products, and 15% household cleaning products. Nonylphenol ethoxylates (NPEs) make up 80% of those APEs on the market (Ying et al., 2002).

Concerns arose in the early 1980's when it was discovered that the breakdown products of these ethoxylates are more toxic to aquatic life than the original compounds (Soares et al., 2008). Interest began as NP's estrogenicity was discovered by accident: the controls in a study with estrogen sensitive tumor cells began mimicking the treatment. The researchers discovered that it was the estrogen "contamination" was 4-nonylphenol in their polystyrene centrifuge tubes (Soto et al., 1991; Gilbert, 2006). They found that nonylphenol caused cell proliferation in human estrogen-sensitive (MCF7) breast tumor cells, and mitotic activity in rat endometrium (Soto et al., 1991).

Based on this information, the European Union began to take action in mid 1990's, phasing out nonylphenolic compounds. This included a ban on the marketing of cosmetics that contained NP, and limited NP concentration to trace amounts (less than 0.1% by weight) in products, "provided that such presence is technically unavoidable in good manufacturing practice and that the product does not cause damage to human health" (Directive 76/768/EEC, REACH).

Canada has also banned the use of NPEs in their detergents, and aquatic safety limits are set to 1.0 ppb in freshwater and 0.7 ppb in seawater (CWQGPAL, 2002).

In the 1990's the United States Environmental Protection Agency (USEPA) performed its own study and found that NPs were better degraded in their own treatment plants (c.f. those in Europe), and decided not to legislate; though many subsequent studies do not support this decision (Soares et al., 2008). Recently, the EPA has developed an action plan for NP and NPEs (RIN 2070-ZA09). This action plan includes the "ongoing voluntary phase-out of NPEs in industrial laundry detergents," as well as rule making under the Toxic Substances Act, sections 4 (Significant New Use Rules) and 5 (Test Rules). This action plan also includes "rulemaking to add NP and NPEs to the Toxics Release Inventory, which would require facilities to report releases of these chemicals to the environment" (RIN 2070-ZA09). While not currently on the list of toxic substances, the EPA's Office of Water has recommended water quality criteria (WQC) for NP. The WQC for freshwater species is 28 ppb acute and 6.6 ppb chronic exposure, and for saltwater species at 7 ppb acute and 1.7 ppb chronic exposure (RIN 2070-ZA09).

Several developing nations continue to use nonylphenolic compounds, such as China, India and several South American countries (Soares et al., 2008).

Alternatives to nonylphenol ethoxylates include other APEs, which can still be used as emulsifiers, dispersants, and surfactants (RIN 2070-ZA09). These include glucose-based carbohydrate derivatives such as alkylpolyglucoside, glucamides, and glucamine oxides (RIN 2070-ZA09). Other alternatives are alcohol ethoxylates, which are less efficient in industry, but degrade faster and therefore may be safer for the environment (Soares et al., 2008). Unfortunately these alternatives have not been around long enough to research the potential effects of breakdown products on organisms (Soares et al., 2008).

Nonylphenol ethoxylates are sold to manufacturers under the trade names Empilan, Igepal, Imbentin, Lissapol, Marlophen, Surfonic, Tergitol, Terric, and Triton (Lee, 1999). However, if APEs or NP are used in the manufacturing process, they are not necessarily listed on the ingredients list.

Physio-chemical Properties

The three types of chemicals covered here are alkylphenol ethoxylates, nonylphenol ethoxylates and

nonylphenol (Table 4). All three are used as surfactants, and differ in their R-groups, level of ethoxylation, and environmental toxicity.

Table 4: Chemical structures of commonly used surfactants.

Alkylphenol Ethoxylate (APE)
$\text{R}-\text{C}_6\text{H}_4-\text{O}(\text{CH}_2\text{CH}_2\text{O})_r\text{H}$
Nonylphenol Ethoxylate (NPE)
$\text{H}_{19}\text{C}_9-\text{C}_6\text{H}_4-\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$
4-Nonylphenol (NP)
$\text{H}_{19}\text{C}_9-\text{C}_6\text{H}_4-\text{OH}$

Alkylphenol ethoxylates are used as surfactants, and break down into shorter chain chemicals in the environment, such as nonylphenol and octylphenol (Ying et al., 2002).

Nonylphenol ethoxylates have a formula of $\text{C}_9\text{H}_{19}-\text{C}_6\text{H}_4\text{O}(\text{CH}_2\text{CH}_2\text{O})_n\text{H}$, with most commercially produced NPEs n is between 6 and 12 ethoxylate groups, though it can range from 1 to 100 (CWQGPAL, 2002).

Nonylphenol is breakdown product from APEs, though it can be produced directly under laboratory conditions. NP is produced in industry “by the alkylation of phenol with

nonene under conditions of acidic catalysis," (Soares et al., 2008). This product is then used to manufacture nonylphenol ethoxylates, by "reacting NP with ethylene oxide under basic conditions," which are less toxic than NP itself (RIN 2070-ZA09).

4-NP has a chemical formula of $C_{15}H_{24}O$. Nonylphenol consists of a phenol ring with a nine-carbon chain in the in either the ortho-, meta-, or para-position around the ring. Para-nonylphenol is in the "4" position on the phenol ring, hence the name 4-nonylphenol. The "nonyl group" can be either branched or straight chain (RIN 2070-ZA09). For this experiment we used branched chain 4-nonylphenol with 99% isomers, (CAS number 84852-15-3), which is the most widely produced NP, making it the most relevant form of NP (RIN 2070-ZA09).

At room temperature, NP is a viscous liquid with a solubility limit of 4.9 (± 0.4) mg/L in water (Brix et al., 2001). NP has a flash point of 141°C, which is why we sterilized our equipment for at least two hours at 300°C.

Nonylphenol and its ethoxylates are amphipathic, with a hydrophobic head (the alkylphenyl moiety) and a hydrophilic tail (the ethoxylate chain). It is these characteristics that make NP a successful industrial surfactant (CWQGPAL, 2002). NPs and NPEs are non-ionic

surfactants, having no electrical charge in water (RIN 2070-ZA09). Non-ionic surfactants are found in,

detergents, cleaners, degreasers, dry cleaning aids, petroleum dispersants, emulsifiers, wetting agents, adhesives, agrochemicals, including indoor pesticides, cosmetics, paper and textile processing formulations, prewash spotters, metalworking fluids, oilfield chemicals, paints and coatings, and dust control agents. (RIN 2070-ZA09)

Endocrine disruption

The endocrine system is the body's hormone signaling system, made up of hormones secreted by endocrine glands, i.e. the hypothalamus and pituitary (Di Giulio and Hinton, 2008). Under normal conditions, these hormones are secreted into the blood to become chemical messengers, which bind cells and will have complex consequences in protein expression (Di Giulio and Hinton, 2008). Endocrine disruption (ED) is the disruption of this system by chemicals that can act like hormones.

Nonylphenol is considered a xenoestrogen, i.e. a man-made industrial chemical that can act like a hormone (Soares et al., 2008). APEs are discharged into wastewater or into the environment, and are probably present in the environment at concentrations above what might induce endocrine disruption (Ying et al., 2002). Xenoestrogens interfere with gonadal, thyroid and adrenocortical

functions in fish and other vertebrate species, which are all regulated by the hypothalamus and pituitary (Di Giulio and Hinton, 2008).

Nonylphenol is a well researched endocrine disruptor, is known to mimic the hormone 17β -oestradiol, and competes for the oestrogen receptor (Soares et al., 2008). Nonylphenol can block the production of natural hormones by inhibiting or stimulating the endocrine system (Soares et al., 2008).

As a hormone, 17β -oestradiol is responsible for the development and maintenance of female sex characteristics, as well as maturation and function of sex organs (Soares et al., 2008). NP also has antiandrogenic activity, which can interfere with the development of male reproductive systems (Lee et al., 2003). This may not be competitive inhibition (as is seen in females where NP outcompetes 17β -oestradiol) but is likely due to activation of the androgen receptor (Lee et al., 2003).

The mode of action of endocrine disruption can be explored by looking at vitellogenin expression, secondary sexual characteristics and histology of the sexual organs (Soares et al., 2008). Vitellogenin is a female egg yolk protein, and its induction in males can indicate endocrine disruption. NP induces vitellogenin in rainbow trout

(*O. mykiss*) at 20.3 mg/l (Jobling et al., 1996), and medaka fish (*Oryzias latipes*) exposed to 0.1 mg/l nonylphenol (Tabata et al., 2001).

Though there is evidence for NP as an endocrine disruptor, our study concentrates on its potential physiological effects. This is because our analysis of the proteomic changes will provide insights into the physiological effects that cannot be detected through the analysis of tissue pathologies. It is interesting to note the gender discrepancy in Morro Bay *C. ios*, which could be a consequence of ED, differential survivorship, or even an artifact of collection methods (Table 3).

Sources

Since nonylphenol is not a naturally occurring chemical, all nonylphenol in the environment can be traced back to manufacturing and anthropogenic activity. Nonylphenol and NPEs can be released into the environment in several ways:

- During production of NPEs
- During production of nonylphenol/formaldehyde
- During production of epoxy resins
- During production of plastic
- In industrial and institutional cleaning products
- During agricultural use
- From emulsion polymerization processes
- In textiles processes including wool scouring
- From paper processes

- From printed circuit boards manufacturing processes
- From metal extraction, refining and processing industry
- During paints, lacquers and varnishes manufacturing processes
- From use in deicer formulations (RIN 2070-ZA09)

The largest contribution to nonylphenol found in the environment includes sewage treatment plants and waste water treatment plants (WWTPs). In 1993 Ahel and Giger found 60-65% of nonylphenolic compounds that enter sewage treatment plant were released into the environment, with most (90%) in association with sludge.

There are several potential nonylphenol inputs into Morro Bay, including agricultural and urban runoff in Chorro and Los Osos Creeks (Figure 2). We were able to sample the sediment in a creek system 100 meters above and below the California Men's Colony WWTP, and found no NP above, but 610 ppb of NP below the outfall. This indicates that WWTPs are breaking down the alkylphenol ethoxylates into nonylphenol as has been documented before (Ying et al., 2002). We also found septic tanks to be substantial environmental sinks for NP, finding over five million ppb of NP in one of the sludge layers taken from a septic tank in the Bayridge Estates, Morro Bay, California (Diehl et al. 2011).

Water

NP can be found at the surface of water at lower concentrations than sediment because the chemical favorably partitions into organic matter (Ahel and Giger, 1993). However, nonylphenol can be found in water, and most studies have focused on anthropogenic origins including WWTPs and urban effluent (Soares et al., 2008). Recall that the WQC in the USA for saltwater species is 7 ppb acute and 1.7 ppb chronic exposure (RIN 2070-ZA09).

Nonylphenol has been found in waters all over the world. NP has been found in lakes in China at 32.8 ppb (Wu et al., 2007), river water in the United States at < 1.0 ppb (Naylor et al., 1992) and in ocean water in Spain at 4.1 ppb (Petrovic et al., 2002).

NP levels were monitored for three years in Italy on the Po River, downstream of industrial and agricultural inputs (Davi and Gnudi 1999). The max NP levels changed from 158 ppb 1994 to 8.8 ppb in 1996; the authors explain the decline was due to a period when NP was being phased out of use in the area.

Water samples from intertidal pools in Morro Bay (which remain on the mudflats during low tide) ranged in 4-NP concentration from undetected (detection limit: 0.1 ppb) to 1.2 ppb. These samples represent 4-NP concentrations

localized in space and time and are within the limits established by the EPA for one hour and 4-day average concentrations of NP in seawater. Liber et al. (1999) provide an approach to estimating the long term average exposure levels of fish to NP via a significant ($p < 0.001$) relationship between the average NP concentration in water (X , $\mu\text{g/L}$) and the measured NP tissue concentration in fish (Y , $\mu\text{g/g ww}$): $\log(Y) = -1.12 + 0.79 \log(X)$. Their field experiment administered 4-NP to littoral enclosures in a Minnesotan pond and assessed the effects on juvenile bluegill sunfish (*Lepomis macrochirus*). Using the average 4-NP concentration in arrow gobies of Morro Bay, 185 ng/g ww, the equation of Liber et al. (1999) estimates the average 4-NP concentration in Morro Bay waters to be 3.09 $\mu\text{g/L}$ (3.09 ppb), which exceeds the EPA 4-day average recommendation. It also exceeds Canadian marine water quality guideline that stipulates the concentration of NP and associated ethoxylates should not exceed 0.7 ppb (Canadian Council, 2002).

Sediment

In sediment nonylphenol has a half life of 60 years (Shang et al. 1999, Soares et al., 2008). Microorganisms within sediment can aid in the biodegradation of NP,

however this is limited by oxygen availability (Soares et al., 2008).

However, under anaerobic conditions, (i.e. the Morro Bay mudflats) NP may not undergo any more transformations and remains attached to sludge solids and partitions favorably into organic materials (Soares et al., 2008).

One interesting consequence of sewage sludge recycling includes the supplementation of nonylphenol to agricultural lands. This can contribute to NP within food crops, as well as NP in runoff (Soares et al., 2008). Nonylphenol also has low mobility in sediments: in 2000 Vogel et al. found that after 730 days, almost 99% of original NP contamination still adhered to the soil particles within the top 30 cm.

The 4-NP concentration in the anaerobic sediments of Morro Bay ranged from undetected to 87 µg/kg, averaging 38.5 µg/kg 4-NP (ww, n=9).

Atmosphere

Nonylphenol is semi-volatile and can be redeposited from the atmosphere by rain and snow. Another contribution of sewage treatment plants includes NP associations with aerosols from plant aerators (Soares et al., 2008).

Interestingly, the USEPA finds that "in the atmosphere, NP will be degraded rapidly by hydroxyl radicals and is not expected to be persistent in air" (RIN 2070-ZA09). However, measurements of NP in air in the from urban and coastal environments in the United States contained 2.2-70 ng/m³, these levels were higher than expected, and higher than those of PCBs and PAHs (Dachs et al. 1999).

Nonylphenol has been measured in indoor environments in air (110 ng/m³) and in dust (2.58 µg/g) in the United States (Rudel et al., 2003). Out of the 120 houses tested, every single house was contaminated with nonylphenol, which was one of the most abundant out of the 89 organic chemicals measured (Rudel et al., 2003).

NP was not measured in the air in Morro Bay in this study.

Effect on humans

Considering that the USEPA estimated the demand for NP to be 380 million pounds for 2010, it is little wonder they are paying attention to novel studies supporting NP's toxicity. The main purpose of the Action Plan is to investigate NP's "PBT" effects on people, i.e. persistent, bioaccumulative, and toxic characteristics (RIN 2070-ZA09).

Potential human endocrine disruption or other physiological effects due to NP exposure has not been studied. However, based on the widespread availability, and bioaccumulative properties, the possible effects of NP on organisms must be assessed. NP is not considered mutagenic in the Ames assay, in vitro chromosomal aberration assay, or in vivo micronucleus assay (RIN 2070-ZA09).

Since NP and NPEs are not currently designated as toxic substances, there are no Permissible Exposure Limits or Recommended Exposure limits in the United States, which accounts for the lack of data on occupational exposure (Di Giulio and Hinton 2004; RIN 2070-ZA09).

Less of a concern for human contact with NP, drinking water treatment plants can remove up to 99% of nonylphenol, depending on treatment method; e.g. ozonation followed by activated carbon filtering with chloronation can remove 95% of nonylphenol (Petrovic et al., 2003; Soares et al., 2008). Treated drinking water has been tested in Spain (.085 ppb) and Germany (.015 ppb), and does not contain nearly as much NP as food and food packaging (Guenther et al., 2002). However, measurements of NP in untreated well water ranged up to 32.9 ppb in Cape Cod, Massachusetts (Rudel et al., 1998).

Nonylphenol enters our food “principally via the use of cleaning agents in the food processing industries, application of pesticides and wax coats on vegetables and from foodstuff packaging material that might leach tris(nonylphenol)phosphate and other nonylphenolic compounds,” (Soares et al., 2008).

Based on diet, Guenther et al. (2002) estimated that the daily intake of NP for an adult is 7.5 µg/day in Germany. Guenther found nonylphenol in all of the 60 food products tested, which were purchased from supermarkets, including items such as beer, pasta, bread, coffee and chocolate. The highest levels found were in butter (14.4 µg/kg), apples (19.4 µg/kg), and tomatoes (18.5 µg/kg).

A similar study in Singapore found alkylphenols in all foods sampled from local supermarkets. They found almost 200 ng g⁻¹ wet weight NP in prawns, and in other seafood such as crab and fish (Basheer et al., 2004).

Another study found high levels in seafood from Italy (Ferrara et al., 2005). Organisms were collected from fishermen and found high average levels of NP in anchovies (988 µg kg⁻¹ fresh weight), mackerels (954 µg kg⁻¹), and red mullets (723 µg kg⁻¹). Based on a diet including sea food, Ferrara estimated that an Italian could intake 12.2 µg day⁻¹ person⁻¹, higher than Guenther et al. (2002) predicted for

Germans. In a follow-up study, Ferrara et al. (2011) looked at NP and other alkylphenols in the adipose tissue of sixteen European adults. They found 4-NP to be the highest concentration of the nine compounds analyzed with an average of 122 ng g⁻¹ fresh weight.

Nonylphenol has been monitored in human breast milk, with the highest levels measured at 56.3 ppb, and with a maximum estimated dose of 3.9 µg/kg/day for an infant (Ademollo et al., 2008). This same study also found a positive correlation between seafood consumptions and levels of NP in breast milk (Ademollo et al., 2008)

Children can come into contact with NP not only through breast feeding, but by crawling on the floor, where they are more likely to come into contact with NP-containing cleaners. Children may be at a higher risk than adults because compared with adults, they drink more water, consume more food, and breathe more air per pound RIN 2070-ZA09). Exposure to NP over several generations can cause "changes in the estrous cycle length, timing of vaginal opening, ovarian weight, and sperm/spermatid count," (RIN 2070-ZA09).

Conversely, A study by Muller et al. (1998) concludes that "the non-occupational exposure to NP does not pose an estrogenic health risk to humans," based on a sample size

of two, with no controls: one human volunteer was given 5mg (67 µg/kg body weight), while the other was given an intravenous dose of 1 mg (14 µg/kg body weight) of NP. However, this study does give us insight into how the human body metabolizes isotope labeled $^{13}\text{C}_6$ -nonylphenol (Muller et al., 1998). Muller found the half-life in blood was 2-3 h, for either administration of NP. Since only 1% of the orally administered NP was found in the subsequent faeces, Muller concludes that rapid hepatic metabolism has removed the NP, while Soares interprets Muller's results in a different way, and believes that the remaining nonylphenol was absorbed in the gastrointestinal tract (Soares et al. 2008).

There is interest in whether solvents, like NP, could be contributing to a worldwide decrease in sperm count. A study in Canada by Cherry et al. (2001) found exposure to organic solvents might be a risk factor for male fertility, looking at a single fertility clinic over twenty years, as well as 10 separate fertility clinics over a four year period. In each case, Cherry concluded that men with high exposure to solvents were likely to have a low active sperm count, and Cherry also states "exposure to organic solvents at work is associated with damage to the nervous system (both acutely and with long term exposure) and to the

skin." Cherry's data does "add considerably to the body of data suggesting that organic exposure to solvents constitutes a hazard for male fertility," note Cherry's total sample size of n=1230, c.f. Muller's n=2 (Muller et al. 1998, Cherry et al. 2001).

The EPA bases potential human health effects of NP on rat toxicity, citing Lowest Observed Adverse Effect Levels (LOAELs; EPA, 2009). LOAELs for systemic toxicity, based on body weight or body weight gain range from 43 to 64 mg/kg-bw/day. Other LOAELs range from 43 to 64 mg/kg-bw/day, including: reproductive toxicity (based on decreases in epididymal sperm density or testicular sperm head counts, increases in estrous cycle length, and decreases in ovarian weights), developmental abnormalities (accelerated vaginal opening in pups), and maternal toxicity (decreased terminal body weights) (RIN 2070-ZA09).

Nonylphenol exposure has been linked to cancer in rats. Those fed a diet of 25 or 250 ppm NP had significantly higher incidences of lung adenoma and carcinoma, c.f. rats not fed any NP, over a 28 week period (Seike et al., 2003). Also, the number of epithelial cells in both the lungs and colon increased over the same period in both NP treatments. Alveolar cells of the 250 ppm fed rats expressed significantly higher levels of proliferating

cell nuclear antigen (an enzyme key to DNA synthesis and repair), and higher levels of 8-OHdG (a DNA adduct indicating oxygen radicals). DNA damage and cell proliferation are both strong indicators of cancer (Seike et al., 2003).

Bevan et al. (2003 and 2006) found several abnormal cellular responses when exposing frog embryos (*Xenopus laevis*) to NP at levels from 10 nM to 10 μ M. One dose at 11 hours (stage

10.5) was substantial enough to see "increased mortality, induced morphologic deformations, increased apoptosis, and altered the deposition and differentiation of neural crest-derived melanocytes in tailbud stage embryos" (Bevan et al., 2003). The tadpoles also had developmental abnormalities (Figure 9) and behavioral abnormalities, including "abnormal swimming patterns and spasticity when

Figure 9: From Bevan et al., 2003: "Figure 2. Representative examples of (A) an embryo maintained in vehicle alone (EtOH) and (B) one maintained in saline containing 1 μ M nonylphenol beginning at stage 10.5 for 48 hr (~stage 37 for control embryos). Crooked backs, kinked tails, poorly developed somites and dorsal fins, and "chicken-beaked" cement glands (CG) were often evident in embryos treated with micromolar concentrations of the estrogenic compounds, E2, octylphenol, or nonylphenol.

touched, [which] may reflect aberrant patterns of sensory or motor inputs," (Bevan et al., 2003).

In a follow up study, Bevan et al. (2006) investigated the effect of NP on *Xenopus* neurons, finding that NP may interfere with neuronal development (specifically neurotrophin-dependent neurite outgrowth) due to bioaccumulation and aberrant steroid signaling. Bevan is adamant that even acute exposure indicates NP may be altering signaling pathways and therefore cell fate in these cell types.

The toxicity of nonylphenol on cell regulatory systems is summed up by Soares (2008), and includes:

1. Capacity to trigger respiratory toxicity in cells, probably by changing the membrane permeability of the mitochondria; EC50 1.8 mg/l (Argese et al., 1994)
2. Adversely affect the active transport of calcium to the sarcoplasmic reticulum in resting skeletal muscle cells; IC50 880-2420 mg/l (Michelangeli et al., 1990; Kirk et al., 2003)
3. Inhibit the growth and differentiation of murine neural stem cells and induced their apoptosis in contrast with other endocrine disruptors; effective concentration N660 mg/l (Kudo et al., 2004)
4. Increase proliferation of mammary gland cells at exposure concentrations of 0.01 mg/day (Colerangle and Roy, 1996)
5. Alter the cell-cycle kinetics by conversion of immature structures to mature structures (Colerangle and Roy, 1996)
6. Produce telemetric associations and chromosomal aberrations (Roy et al., 1998).

Here in Morro Bay, while not the focus of our investigation, we have found nonylphenol in virtually every

organism, environment and even human industrial product tested so far, echoing the results of former research. We have found NP in mussels and oysters intended for human consumption: 313 ng/g (n=3) in *Mytilus californianus*, and 446 ng/g (n=2) in *Crassostrea gigas* (both wet weights, Diehl et al., 2011). We have also found NP in toilet paper, the highest level in Charmin Ultra Strong, at 2790 ng/g ww, perhaps due to its patented manufacturing methods or the type of wood utilized. We found NP in tampons, with the highest level found in Tampax brand at 190 ng/g ww. While the levels in tampons are lower than toilet paper, consider that their use by women places this endocrine disruptor in direct contact with mucosal membranes.

Nonylphenol has been discovered in all compounds tested so far. It can be found in the food we eat, the water we drink, and the packaging our food comes in. NP is in makeup, lotions and household cleaners. It's even in the atmosphere. Comparing NP to DDT, banned in 1972, both are hydrophobic, persistent and xenobiotic. Considering we found isomers of DDT in our sediment samples from Morro Bay in 2009, we should consider the long term effects, e.g. bioaccumulation, of not acting to reduce levels of NP right now. In summary, "The effects of nonylphenol are very

diverse and unpredictable as a consequence of its ability to initiate numerous responses," (Soares et al., 2008).

Tools

Proteomics is a powerful tool for investigating how a cell or organism directly deals with perturbation, e.g. thermal, osmotic, or chemical stress. This field can contribute to previously unknown cellular effects, and is an influential tool for generating hypotheses regarding how the biology of an organism is affected by a stressor.

In the following sections the methods of proteomics are explored along with its direct application seen with fish, and how "ecotoxicoproteomics" combines the best of ecology, toxicology, and proteomics to investigate complex biological processes.

Proteomics

Figure 10: Two-Dimensional Gel. 100ug arrow goby liver protein. 4-7pH IPG strips, 11% PAGE, SYPRO ruby stain.

Proteomics is a field in which the expressed proteins of an organism or tissue are investigated. Proteins are the functional units of a cell, and their study can provide insight into how an organism may be dealing with perturbations such as changes in environment or stress. This systems biology approach is discovery based (c.f. hypothesis driven), resulting in a suite of testable hypotheses concerning the molecular workings of observed phenomena (Lovric, 2011; Tomanek and Zuzow, 2010).

In contrast with traditional genetics and biochemistry, in which select genes and/or their encoded

proteins are deliberately examined, proteomics investigates global changes in protein expression. The so called "-omics" technologies have similar goals of characterizing cellular and organismal response to perturbations. Genomics involves the sequencing and mapping of genes, as well as genetic inheritance patterns. With the completion of the Human Genome Project in 2003, it became evident that knowing the genetic code was only the first step to understanding the multifaceted connection from genes, to cells and organisms.

Additionally, with the complex synthesis, processing, localization, and degradation of proteins, transcriptomics (or functional genomics) may not yield a complete picture. Studying the mRNA message via microarrays may exclude important biological information, e.g. if protein abundances don't parallel changes in transcript levels (Berth et al., 2007). Furthermore, the correlation between mRNA and actual protein expression is "lower than 0.5, because the rates of degradation of individual mRNAs and proteins differ, and because many proteins are modified after they have been translated, so that one mRNA can give rise to more than one protein," (Abbott, 1999).

A good molecular indicator must be sensitive to minute changes in environment, have a fast response time, and have

a prolonged duration with the cell (Feder and Walser, 2005). While the genetic blueprint remains stagnant and the mRNA message may not be a direct indicator, even subtle environmental changes can alter protein expression. Protein activity may be the best indicator of survival and fitness (Feder and Walser, 2005). Proteomics allows for the study of simultaneous changes in expression of hundreds to thousands of proteins, as well as analysis of post-translational modifications, which are important for cell signaling. Consequently, investigating changes in protein abundance can tell us how an organism is directly and immediately coping with changes in environment due to stress or environmental toxins, and utilizing gel technology gives us a snapshot in time of this cellular response.

A common technique in proteomic studies, which is used in this experiment, is two-dimensional gel electrophoresis (2DGE; Figure 10) and mass spectrometry (MS; Figure 14). This method involves separating proteins by their isoelectric focusing point (pI) and their molecular weight, and subsequent identification via mass spectrometry. Two-dimensional gel electrophoresis was first introduced in 1970 by Kendrick and Margolis, though the field was not

termed “proteomics” until 1996 by Dr. Marc R. Wilkins (Lemos et al., 2010).

Methodology

The Environmental Proteomics Laboratory uses two-dimensional gel electrophoresis¹ to visualize expressed proteins on a gel. The procedure of 2DGE can be broken into four main steps: protein preparation, separation, quantification and identification. Because the entire workflow can be very specific to the type of organism or laboratory, there is no “universal” sample preparation, and each protocol must be optimized (Görg et al., 2004).

Proteomics techniques can take time and precision to master, nevertheless in “a suitably equipped and experienced lab environment, 2-D gels are easy to handle, and they can be produced in a highly parallelized way,” (Berth et al., 2007).

Protein preparation

It is vital for proteomics that samples are first prepared accurately and consistently. This step determines the success of the subsequent separation technique and provides a foundation for yielding accurate and publishable

¹For full materials and methods please refer to section in manuscript, page 116.

results, as preparation affects the resolution, quality, and reproducibility of gels. It is fundamental that proteins are correctly denatured, disaggregated, reduced and solubilized before further processing (Görg et al., 2004).

Figure 11: 2DGE workflow, A) Experiment, B) Protein preparation including homogenization, precipitation and quantification, and protein separation including C) First dimension isoelectric focusing (biorad) and D) Second dimension SDS-PAGE (biorad).

After completing an experiment, all tissues must be frozen quickly, i.e. with liquid nitrogen or (used here) dry ice (Figure 11A; Berth et al., 2007). This preserves cellular activity at the conclusion of the experiment.

Freezing samples quickly also prevents protease activity from destroying the proteome.

Full sample preparation includes homogenization, precipitation and quantification; and is necessary for cell disruption and lysis, protein extraction and solubilization, and contaminant removal (Figure 11B; GE Healthcare, 2005). First the organism is dissected and the target tissue(s) removed (*C. ios* liver). These tissues are homogenized via grinding to facilitate cell disruption, along with homogenization buffer. This buffer denatures proteins by disrupting the structure of water via a chaotrope like urea, improves solubility of membrane proteins with thiourea, stabilizes pH utilizing tris base, cleaves disulfide bridges with DTT, contains ampholytes which help proteins move to their correct pH, and a zwitterionic detergent which disrupts cell membranes and helps solubilize integral membrane proteins like ASB-14 (Table 5). A final centrifugation helps to remove large insoluble debris at this step (GE Healthcare, 2005). While some laboratories add proteases during the homogenization step to prevent enzyme activity within the sample, we have found that the high concentration of urea is suitable for this purpose (Shaw et al., 2003).

Table 5: Chemicals used in protein preparation for 2DGE

Chemical	Purpose	Buffer / Step
Acrylamide	Gel matrix used to separate proteins	Second Dimension
Ampholytes	Help proteins move to their correct pH	Homogenization Buffer IPG Rehydration Buffer
ASB-14: Amidosulfo- betaine-14	A zwitterionic detergent which disrupts cell membranes and helps solubilizers integral membrane proteins	Homogenization Buffer
CHAPS 3-[(3- Cholamidopropyl) dimethylammonio]- 1-propanesulfonate hydrate	A zwitterionic detergent used in solubilizing hydrophobic and integral membrane proteins	IPG Rehydration Buffer
DTE Dithioerythritol	A reducing agent similar to DTT	IPG Rehydration Buffer
DTT Dithiothreitol	Cleaves disulfide bridges	Homogenization Buffer Equilibration Buffer
Glycerol	Stabilizes established gradient within IEF strip	Equilibration Buffer
Iodoacetamide	Alkylation of remaining DTT	Equilibration Buffer
Nonidet P-40 substitute	Non-ionic detergent for solubilizing membrane proteins and preventing hydrophobic interactions	IPG Rehydration Buffer
SDS sodium-dodecyl- sulfate	Ionic detergent to maintain protein solubility	Equilibration Buffer
TCA/Acetone	Removal of interfering substances, protein enrichment	Precipitation
Thiourea	Improves solubility of membrane proteins	Homogenization Buffer IPG Rehydration Buffer
Tris base	pH stabilizer	Homogenization Buffer
Tris-HCl pH 8.8	Establishes pH for SDS-PAGE	Equilibration Buffer
Urea	Chaotrope: disrupts the structure of water	Homogenization Buffer IPG Rehydration Buffer Equilibration Buffer

While urea is very efficient at denaturing proteins, there are some potential drawbacks to using this compound. First, urea makes solubilization of hydrophobic membrane proteins difficult, though thiourea is useful to counteract this (Görg et al., 2004). Second, urea can potentially cause carbamylation of proteins at temperatures above 37°C due to its inherent equilibrium with ammonium (iso) cyanate (Görg et al., 2004). However, this may not be a problem unless samples remain above room temperature for more than 24 hours (Shaw et al., 2003). This potential problem can best be prevented by making fresh buffers and keeping them at cold temperatures, and returning samples to the proper temperatures immediately after processing. DTT has also been shown to scavenge cyanate (Shaw et al., 2003). Urea is also neutrally charged, so it will not migrate and disrupt isoelectric focusing (Shaw et al., 2003).

Post homogenization there are still several interfering substances remaining in the sample such as salts, lipids, nucleic acids and polysaccharides which are removed via protein precipitation. Salts can cause a multitude of problems, especially in the first dimension. A sample contaminated with salt can cause IPG strips to swell via electroendosmosis which may also cause proteins to precipitate and be lost (Görg et al., 2004; Shaw et al.,

2003). Too much salt will also prolong the first dimension, due to increased conductivity (Görg et al., 2004). Lipids can cause hydrophobic interactions, interact with membrane proteins, and form complexes with detergents which "consume" the detergents (Görg et al., 2004). Also, lipids which are bound to proteins will alter their charge and mass, and can render proteins insoluble so that they are lost in the first dimension (Shaw et al., 2003). Nucleic acids are negatively charged and migrate towards the anode, interfering with isoelectric focusing, and can bind proteins, which prevents them from focusing properly (Shaw et al., 2003). Polysaccharides can clog gel pores and some charged polysaccharides will interact with proteins, resulting in streaks in the 2D gel (Görg et al., 2004).

These contaminants can be removed through several methods, e.g. a protein precipitation. A 10% trichloroacetic acid (TCA)/acetone outcompetes proteins to form bonds with water which results in proteins precipitating from solution. A 100% acetone wash will then wash away the remaining TCA from the protein pellet. Lipids are also removed by acetone. In this precipitation step, the protein is partly purified and most of the interfering substances like salts and lipids are removed (Görg et al.,

2004). Unfortunately, some protein will always be lost during the precipitation step due to insufficient resolubilization of proteins, however removal of interfering substances is vital for 2DGE (Görg et al., 2004). The TCA/acetone precipitation steps are useful for enriching alkaline proteins, e.g. ribosomal proteins, as well as further inactivating proteases (Görg et al., 2004).

Next the proteins must be denatured and reduced, while still retaining their natural charge and size. Samples are suspended in a working IPG rehydration buffer which is similar to the homogenization buffer in that it also denatures proteins by disrupting the structure of water with a chaotrope like urea, improves membrane protein solubility, and contains ampholytes which help proteins move to their correct pH. In addition, working IPG rehydration buffer contains: nonidet P-40 substitute which is a non-ionic detergent for solubilizing membrane proteins and preventing hydrophobic interactions; CHAPS, a zwitterionic detergent used in solubilizing hydrophobic and integral membrane proteins; and DTE, a reducing agent similar to DTT (Table 5; Görg et al., 2004). This step is also followed by centrifugation to remove any remaining debris, especially nucleic acids (GE Healthcare, 2005).

Next, to quantify each sample, a protein concentration assay is performed. Here, a 2-D Quant Kit (GE Healthcare) is used, according to manufacturer's specifications, in which the concentration of unknown samples is compared to a linear standard of known concentration. This is a colorimetric based kit where proteins bind to copper within a solution. A higher concentration of proteins will bind more copper, which removes the copper from solution, i.e. a lighter solution color indicates a more concentrated sample (2-D Quant Kit Instruction Manual). The unbound copper is measured via a spectrometer and sample absorbance is compared with a standard, and used to calculate the concentration of protein in each sample. These results are used later so that a consistent amount of proteins is added to each gel. Inconsistencies in protein load can cause inconsistencies between gels, i.e. low abundance proteins appearing or disappearing.

Protein separation

The next step is the separation of proteins according to their isoelectric focusing point (pI), also known as the first dimension (Figure 11C). For the first dimension, samples are diluted into working IPG rehydration buffer, for a final concentration of 100µg of protein, based on the

aforementioned protein concentration assay. This solution is applied to a "gel strip." First dimension strips can be linear or nonlinear, have a pH range that is "wide (e.g. IPG 3-12), medium (e.g. IPG 4-7), narrow (e.g. 4.4-5.5) and/or ultra-narrow (e.g. IPG 4.9-5.3)," come in a variety of lengths (e.g. 11cm or 24cm), and are nowadays generally purchased rather than poured (Görg et al., 2004). Because the inherent pH gradient is "immobilized," when an electric current is applied through this strip, proteins migrate to their isoelectric

focusing point (pI). This is the pH at which the proteins have a neutral pI (Figure 12; GE Healthcare, 2005). Too little time to focus will

Figure 12: Isoelectric focusing

result in horizontal streaking, while too much focusing will result in electroendosmosis (Görg et al., 2004). For this project 11cm pH 4-7 IPG strips were used, as the separation of proteins between pH4-7 yields the greatest diversity while maintaining high resolution.

In preparation for second dimension gel electrophoresis, the IEF strips are equilibrated in equilibration buffer (Figure 11D). This buffer helps

establishes pH for SDS-PAGE with Tris-HCl, pH 8.8; maintains denatured proteins with urea; stabilizes established gradient within IEF strip through glycerol; and contains SDS, an ionic detergent to maintain protein solubility. Strips are equilibrated twice, first in equilibration buffer with the addition of DTT to reduce disulfide bonds, and second in equilibration with iodoacetamide, which is used for alkylation of remaining DTT (Table 5; Görg et al., 2004). Strips are then placed on a sodium-dodecyl-sulfate polyacrylamide gel for electrophoresis (SDS-PAGE). This step separates the proteins by molecular mass, with the smallest proteins moving the quickest through the acrylamide matrix, and thus the high molecular mass proteins are at the "top" of the gel, and the low molecular mass proteins at the bottom (Figure 10; GE Healthcare, 2005). In this case we used an 11% acrylamide gel matrix. Higher percent gels with more acrylamide can be used to better resolve low molecular mass proteins (GE Healthcare, 2005).

After the second dimension proteins are visualized on the gel via staining. There are a variety of commercial stains available including visible, florescent, and metal stains. Visible stains include the popular Colloidal Coomassie Blue, which is easy to use, inexpensive, and

compatible with most forms of mass spectrometry. Coomassie is poor at resolving low abundance proteins, i.e. has a low sensitivity (200-500ng protein/spot) but has a good dynamic range (Görg et al., 2004). Silver stains are the most sensitive (0.1ng protein/spot), though with a limited dynamic range. Results from silver staining are not as reproducible as those with Coomassie. The more sensitive silver stains are incompatible with MS, and tend to be the most expensive of stains, though the latest silver stains attempt to improve upon this (GE Healthcare, 2005; Görg et al., 2004). Fluorescent stains, e.g. SYPRO Ruby (used here) yield "better and more confident results in terms of sensitivity [1-2ng protein/spot] and dynamic range," (Görg et al., 2004). Not only do these stains tend to be expensive, but they require an additional investment of expensive compatible equipment, such as scanners and spot pickers. Advantages of fluorescent stains include reducing variation between gels, and compatibility with subsequent mass spectrometry (Forné et al., 2010).

Once gels have been sufficiently stained, they must be scanned with an appropriate scanner, e.g. a fluorescent scanner for a fluorescent stain. The quality and resolution of the final image is determined by the sensitivity of the scanner, so care should be taken with this step to make

sure that filters and settings are correct and constant throughout the project (Berth et al., 2007). Any changes to the image after scanning, such as image “clean up” or other manipulations can result in loss of data, and therefore should be minimized. In essence, the gel should be scanned once, using the appropriate settings, and imported directly into the gel image analysis software.

Protein quantification

After staining and scanning, the gel images are analyzed for differentially expressed proteins via gel image analysis software (Figure 13). The goal at this step is to create and analyze a “fusion image” or proteome map, which includes all proteins within a project for expression analysis; this process could not be completed without state of the art software and “this process may be quite laborious and time-consuming” (Görg et al., 2004).

In order for this step to accurately calculate and compare protein expression, the workflow leading up to quantification must be consistent especially in protein load and staining protocol.

Figure 13: Protein quantification with gel image analysis software, including A) image warping, B) image fusion, C) spot detecting and editing, D) application of consensus spot pattern, and E) extracting expression profiles (Berth et al., 2007).

Popular gel image analysis software includes PDQuest (Bio-Rad, Hercules, CA), Melanie III (Genebio, Geneva, Switzerland) and, used in this project, Delta2D (DECODON, Greifswald, Germany). There are several advantages to Delta2D over other systems, e.g. removal of distortions, as well as the ability to warp gels before finding spot boundaries (Berth et al., 2007).

First, all gel images are imported into the program and grouped based on treatment and time. These gels are “warped” to one another so that the same protein spot on one gel is matched to its counterpart on every other gel.

This is done manually between each gel pair for accuracy and mismatches must be carefully checked and edited manually (Figure 13A; Görg et al., 2004). Images must be warped due to "variation in the spot positions between gels," (Berth et al., 2007), which are not due to error or bias, but acknowledges that small variations within gel runs may produce slight differences between gels. These must be corrected for, because "even a very experienced experimenter will not be able to produce 'perfect' gels whose spot patterns show exact congruency," (Berth et al., 2007). These errors are localized and can be fixed in the gel image software by a trained eye via warping.

Next, a fusion image is generated, which is a representative image of all the proteins on each gel within the project (Figure 13B). On this fusion image each spot is then detected to generate a "consensus spot pattern" which represents how the proteins are distributed throughout the experiment (Figure 13C).

This spot pattern is then applied to all other gels within the experiment (Figure 13D). Because of the earlier warping, this spot pattern should fit completely onto all of the other gels, creating a proteome map. Next, the normalized volume of each protein is detected, based on spot volume/total spot volume ratio within a single gel.

This process should remove any gel-to-gel variation, and is another example of how important it is to add a consistent load of protein in the first dimension step. Next, statistical analysis of differential protein expression is preformed and expression profiles are generated. This information is often displayed graphically in a "heat map" indicating the relative up- and down-regulation of proteins found in the proteome map (Figure 13E). Each step in this entire process is scrutinized in order to maximize reliability of results.

There are several statistical tools available in the analysis software. For simple control versus treatment experiments, a Student's t-test can be employed, to detect the proteins that are changing due to treatment. Spot densities correspond with protein expression: a higher density spot indicates more protein expression in the organism. Experiments with more complex treatment levels can utilize one- or two-way ANOVAs. The aforementioned tests are relatively robust to non-normal data distributions. However the use of permutations may be necessary because spot volume variances are unequal along with an inherent non-normal distribution (Ludbrook and Dudley, 1998). Permutations of the data construct a normal

distribution, which is preferred in proteomic studies over t - or F -statistics (Ludbrook and Dudley, 1998).

It is a useful statistical tool to correct for the false discovery rate (FDR). There is a positive correlation between the size of a data set and the probability of making a type I error, i.e. falsely rejecting a null hypothesis (Ludbrook and Dudley, 1998). Furthermore, with large data sets of potentially thousands of proteins, even with a low p -value some proteins will still be flagged as significant, though these proteins will be false discoveries (Ludbrook and Dudley, 1998). An FDR "of 5% means that among all features called significant, 5% of these are truly null on average" (Storey and Tibshirani, 2003).

The major drawback to controlling the FDR is the potential of ending up with such strict statistical parameters as to restrict the number of significant proteins to zero. Thus a compromise must be established between acquiring data that is either too lenient and false, or too restricted and therefore not biologically accurate or meaningful. With large proteomic data sets, statistical analyses are not standardized across the field, which leads to the necessity of transparency when reporting statistical methods.

In this experiment normalized spot volumes were analyzed using an analysis of variance of time x treatment (two-way ANOVA). A null distribution was generated using 1000 permutations and a p-value of 0.01 was used to limit the number of false positives instead of using a multiple-comparison correction.

Once a set of proteins is deemed to have significant differential expression due to treatment, hierarchical clustering can be used to arrange the proteins into clusters based on similarities in their expression profiles (Zvelebil and Baum, 2008). This allows for the categorization of proteins that show similar changes in their abundance across treatment groups, which may suggest that they belong to the same cellular pathway (Zvelebil and Baum, 2008). This analysis becomes more meaningful once the protein identities have been determined.

Protein identification

The next step in the proteomic pathway is to identify these proteins of interest, which is typically achieved via a tryptic digest followed by mass spectrometry. The contribution of mass spectrometry to proteomics cannot be overstated, and was the basis for Nobel prize for chemistry awarded in 2002 (Nobel prize website). Without MS, data

from 2DGE would be rendered useless without identification of proteins.

All MS machines aid in the fragmentation of peptide bonds, and popular models include matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and surface-enhanced laser desorption/ionization (SELDI). The first two types use "soft-ionization" techniques that allow for the transfer of intact ionized peptides into a gas-phase, while preventing excessive fragmentation (Domon and Aebersold, 2006). SELDI can be employed for complex biological samples and utilizes a protein chip and MS to explore unidentified expression profiles (Tang et al. 2004). Mass spectrometry is extremely sensitive e.g. can detect femtomole to attomole concentrations of peptides; is high throughput; and can detect post translational modifications such as phosphorylation (Görg et al., 2004).

In preparation for identification, proteins of interest are excised directly from gels via manual spot cutting for stains in the visual range, or via a spot cutter which can detect florescent stain. Once protein spots are removed, they are digested with an enzyme, e.g. trypsin, which cleaves proteins on the carboxyl side of lysine or arginine. Because trypsin cleaves proteins in a

known pattern, the resulting peptide fragments can be matched to peptides in a protein database for identification, after preparation for MS.

Figure 14: Mass Spectrometer A) Environmental Proteomics Laboratory MALDI-TOF/TOF, B) MALDI-TOF/TOF and LIFT diagram (Suckau et al. 2003).

Digested proteins are embedded into a sample matrix when plated onto the sample target. The target is then placed into the mass spectrometer, in this case the matrix-assisted laser desorption/ ionization time-of-flight/time-of-flight (MALDI-TOF/TOF; Figure 14; Bruker Daltonics Inc., Billerica, MA, USA). The matrix is targeted by a laser, leading to its disruption and the formation of positively charged peptides. These charged peptides are accelerated and move through the mass analyzer to a distinct area on the ion detector which records the peptide's mass to charge ratio (m/z). More specifically,

MALDI-TOF/TOF instruments are characterized by a co-linear arrangement of two TOF mass analyzers, each equipped with an ion source that allows acceleration and focusing of the ions. In TOF1 the analyte ions are accelerated, precursor ions

are selected and fragmented. Fragment ions are allowed to proceed to the 'source' of TOF2, where they are accelerated and mass analyzed. (Suckau et al. 2003)

The information from TOF1 is conveyed in graphical form in a peptide mass fingerprint or PMF (Figure 15). Each protein has a unique PMF, which can be searched within databases of known proteins. Databases of protein sequences are invaluable to this process, and can include expressed sequence tag libraries (EST libraries) with cDNA sequences. Popular databases include National Center for Biotechnology Information (NCBI), the Expert Protein Analysis System (ExPASy), and Matrix Science Database (MSDB or MASCOT).

Identification via PMF is efficient for "species whose genomes are relatively small, completely sequenced, and well annotated, but not so reliable for organisms whose genomes have not been completed" (Görg et al., 2004). Several fish have sequenced and annotated genomes, such as the zebrafish (*Danio rerio*), however *C. ios* is not sequenced. Protein identification of unsequenced organisms can be difficult and time consuming, and requires skill and patience for successful identifications.

For quality control purposes, each protein-database match is assigned a score which represents the probability that the given match is due to random chance (Pappin et al., 1993; Drabik et al., 2008). The MASCOT server utilizes a molecular weight search (MOWSE) algorithm which reports scores as $-10 \cdot \text{LOG}_{10}(P)$, where P represents the absolute probability that the observed match is a random event (Pappin et al., 1993; Perkins et al., 1999). A protein is considered identified if two criteria are met: 1) the score is above the significance threshold (p-value 0.05) and 2) two or more peptide fragments are matched (Pappin et al., 1993). For *C. ios* proteins, a MOWSE score above 36 in the

MSDB Actinopterygii database is considered significant. Further, a higher MOWSE score indicates a low probability that the match is false (Pappin et al., 1993; Perkins et al., 1999).

To aid in identification, peptides in high abundance can be further broken up in a process called tandem mass spectrometry which is also known as MS/MS or LIFT in the TOF2 compartment (Figure 14B; Figure 15, inset). In the MALDI-TOF-TOF, a "lift" apparatus raises the potential energy of the peptide fragments (Suckau et al., 2003). Combining PMF and LIFT information can lead to an increase in protein identification, an 11% increase in identification rate was seen for Suckau (2003). Unfortunately this process "cannot be readily automated, and considerable time and expertise are required for interpreting the MS/MS spectra," (Görg et al., 2004).

It is only after identification of proteins of interest that hypotheses are formed as to their involvement in the cellular response. These testable hypotheses concern the molecular workings of cellular responses to perturbations (Tomanek and Zuzow, 2010). Hierarchical clustering is very useful in forming these hypotheses, because the proteins are already organized by similarities in their expression profiles, possibly indicating clustered

proteins may belong to the same cellular pathway (Zvelebil and Baum, 2008).

Limitations

As with any methodology in science, proteomics is subject to limitations based on equipment, user error and reality.

A recent topic of discussion in the proteomics literature is whether significant proteins are an artifact of the methodology vs. biologically informative, i.e. in response to stressful conditions (Wang et al., 2009; Petrak et al., 2008). Proteomic studies of stress often result in similar proteins being expressed, indicating that these proteins are possibly either selected for by the sample preparation process or are a type of universal cellular stress response. These proteins include "several cytoskeletal and stress proteins, proteasome subunits, glycolytic enzymes, elongation factors, and heterogenous ribonucleoprotein particle subunits or glutathione transferases," as well as DNA and protein repair enzymes (Petrak et al., 2008). This can be informative in many ways: first, when identifying proteins such as these, it is a simple indication that stress is occurring. Second, this universal cell stress response is in direct contrast with

the idea of biomarkers, where precise protein expression can indicate the biological response to a specific stressor; this can aid in the accurate identification of biomarkers by ruling out said proteins. Third, lists of commonly identified stress proteins can help prevent “unjustified over interpretation of 2-DE- based studies,” (Petrak et al., 2008). And fourth (though unrelated to cellular stress response), knowing the limits of the two-dimensional and MALDI approach can lead to technique improvement.

Wang et al. (2009) also investigated the phenomenon technical bias vs. true cellular stress response. This study concluded that it is unlikely that a protein would be significant based solely on its abundance or size: “because stress response is highly conserved, the concept of a minimal cellular stress proteome has been proposed.... Cellular stress response can be the universal reason as to why these proteins are generally expressed differentially,” (Wang et al., 2009). A highly successful cellular stress response would be selected for and passed on to generations, which is why we would see similar patterns in stress proteomics, regardless of the stressor (Kültz, 2005).

Already stated in this section are problems that can result from urea, and interfering substances e.g. salt. These problems can be resolved with a good enrichment protocol. There is also potential loss of protein during each step in the workflow. Many of the "problems" associated with 2DGE can be conquered with attention and practice.

Additional limits of 2DGE preparation include the inability to observe the entire proteome, ranging from 10-90% based on methodology employed. Also, it can be difficult to detect extremes including very acidic or basic proteins, because they are often outside the resolving range (pH 4-7 was used here); low abundance proteins, which may not visualize on gels; and membrane proteins which are difficult to maintain in solution. In the past, proteomics has been criticized for its lack of reproducibility. While this may be true for new investigators and organisms in the field, taking the time to learn solid general practices followed by a period of optimization is the key to reproducibility and quality gels.

There are different suites of proteomics tools available, including, labeling techniques such as iTRAQ and DIGE, as well as semi-gel-free systems, and shotgun gel-free liquid chromatography methods (Berth et al., 2007).

Proteins can be labeled with tags such as isotope-coded affinity tags (ICATs) or isobaric tags for relative and absolute quantitation (iTRAQ), followed by subsequent MS (Fenselau, 2007). An advantage of iTRAQ is that quantitative comparison of four or more protein pools can be performed simultaneously (Fenselau, 2007). Using covalent binding dyes also allows for several samples to be separated and visualized with 2DGE. Difference gel electrophoresis (DIGE) utilizes Cy dyes: Cy2, Cy3, and Cy5 are attached to separate samples, which are subsequently pooled and run on a single gel. The gel is scanned at different settings to yield "2-D patterns with perfect positional identity" (Berth et al., 2007).

Semi-gel-free systems, and shotgun gel-free liquid chromatography methods do not use SDS-PAGE, but instead utilize high performance liquid chromatography (HPLC) and MS to identify proteins in a complex sample (Berth et al., 2007). These techniques are able to better resolve low abundance and hydrophobic membrane proteins, as well as extreme acidic and basic proteins (Rabilloud, 2002).

Given some limitations, protein activity is still the best indicator for survival and fitness (Feder and Walser, 2005). Proteins are the functional units of a cell, and their study can provide insight into how an organism may be

dealing with perturbations such as changes in environment or stress. Consequently, investigating changes in protein abundance can tell us how an organism is directly and immediately coping with changes in environment due to stress or environmental toxins, and utilizing 2DGE gives us a snapshot in time of this cellular response.

An advantage in the field, proteomics is currently the "only technique that can be routinely applied for parallel quantitative expression profiling of large sets of complex," data (Görg et al., 2004). 2DGE can resolve over 5000 expressed proteins, and detect down to less than one ng of protein per spot, and allows the exploration of changes in expression level, isoforms and post translational modifications (Görg et al., 2004).

Types of Research

Most proteomic studies can be divided into three categories, based on the complexity of data presented (Tomanek, 2011). The first level deals solely with the unique protein expression profile in response to pollutant exposure without protein identification. This type of study can be furthered with SELDI-MS. Second are studies that identify these proteins of interest, which can lead to hypotheses as to how/why a cell/organism may be reacting to

a certain perturbation. This is facilitated with either MALDI- or ESI-MS (Tomanek, 2011). The third focuses on post translational modification changes, and can offer insight into protein processing, accomplished by the MALDI-TOF/TOF. In any method, researchers are looking for so called protein expression signatures (PESs) which are unique and reflective of the original experimental perturbation.

Proteomics of Fishes

Proteomics of fishes has been gaining interest in the scientific community, especially in the last decade (Forné et al., 2010). The sequencing of *D. rerio* has established the freshwater zebrafish as a powerful emerging model organism, and allows for identification of proteins and post translational modifications. Proteomic studies with fish have focused on three main topics: development, including embryogenesis and organogenesis; models for drug discovery and human diseases; and aquaculture and toxicity studies.

The zebrafish has contributed significantly to the field of developmental biology (Forné et al., 2010). Early stages of embryo development have been mapped via proteomics, noting differential protein expression between the stages (Lucitt et al., 2008). Similarly, phases of growth and differentiation of zebrafish ovarian follicles have been investigated, yielding a map of the vitellogenin-derived yolk proteins, including post translational modifications and protein accumulation over stages (Ziv et al., 2008). Focusing on PTMs, O-linked b-N-acetylglucosamine and phosphorylation of Ezrin 2 (Villin-2), are both important in *D. rerio* development (Webster et al., 2009; Link et al., 2006). These types of proteomic

studies in conjunction with other model organisms can lead to insights into morphogenesis, oogenesis, and early development (Forné et al., 2010).

Despite its popularity, there are currently no proteomic studies utilizing the zebrafish in biomedical research (Forné et al., 2010). Alternatively, the green swordtail (*Xiphophorus* sp.) has been used in proteomic studies investigating human melanoma and malignant tumors (Meierjohann et al., 2004). The Atlantic salmon (*Salmo salar*) has been used to investigate post-injury neuronal tissue regeneration via proteomics (Zupanc et al., 2006). Similarly, the electrical ray (*Torpedo californica*) was used to investigate synapse transmission at the neuromuscular junction (Nazarian et al., 2007). A PTM study focused on stress and o-acetylation of sialic acids in N-glycans of *S. salar*, and found that the PESs could be altered by long term stress (Liu et al., 2008). Furthermore, the field of glycomics is promising in biomarker development for disease and cancer (Liu et al., 2008; Forné et al., 2010)

With nearly half the fish for human consumption coming from commercial and aquaculture industries, there has been an increase in the number of proteomic studies in the past fifty years. Most research in this industry focuses on

increasing the quality and yield of the product, concentrating on impacts of infection, stress and density, and gonadal and fertility aspects (Forné et al., 2010). Susceptibility of farmed channel catfish (*Ictalurus punctatus*) to infection via enteric septicemia has been investigated via proteomics, identifying proteins that contribute to disease resistance (Booth and Bilodeau-Bourgeois, 2009). Research with *S. salar* has investigated the effects of density stress, finding protein expression signatures (PESs) specific to density level (Provan et al., 2006). Broods of cultured European sea bass (*Dicentrarchus labrax*) are often genetically controlled via cryopreserved sperm. A proteomic study found that this method not only causes degradation of sperm proteins, thereby decreasing sperm motility; but also may be responsible for the low hatch rate seen in those eggs fertilized with the cryopreserved sperm (Zilli et al., 2005). A proteomic study identified proteins involved with muscle growth in the yellow perch (*Perca flavescens*), with the purpose of increasing muscle mass and therefore profitability of fish in commercial aquaculture (Reddish et al., 2008).

The final category of fish proteomics has focused on toxicity, and the emerging field of ecotoxicoproteomics,

involving what proteins can tell us about the effects of pollutants, and is the subject of the next section.

Ecotoxicoproteomics

Toxicology is the study of adverse effects of chemicals (Di Giulio and Hinton, 2008). While traditional toxicology focused on poisons and their effects on humans, in the 1970's the term "ecotoxicology" was coined, focusing on how chemical pollutants effect "free-living flora and fauna and their populations, communities, and supporting ecosystems" (Di Giulio and Hinton, 2008).

One shortfall of ecotoxicology is that the biological effects of pollutants are often poorly understood. This information is especially lacking for emerging contaminants, such as nonylphenol (Tomanek, 2011). Supplementing ecotoxicological studies with proteomics "is a gigantic step since the proteomic profiles embody the link between effects at both the molecular level and the whole organism level, given that proteins are the first functional stage directly or indirectly affected by toxicants," (Lemos et al., 2010).

With the marriage of these two fields, comes the term "ecotoxicoproteomics," which is "an investigation looking at the impact of toxins on a sentinel species using proteomics," (Taylor, 2007). Besides looking for biomarkers of toxicity, ecotoxicoproteomics also looks at the mode of action (MoA), which can provide direct insight

into the chemical mechanism of toxicity (Sarkar et al., 2006; Tomanek, 2011). Knowing the MoA of a chemical will prove advantageous in risk assessment of emerging contaminants, and could become even more informative when combined with a set of biomarkers.

In order to keep this section concise and relevant, ecotoxicoproteomic studies utilizing marine organisms, mainly fish, and/or studies with relevant chemicals will be covered forthwith. Again, there are three categories of complexity in proteomics studies. First are studies that deal solely with the unique protein expression profile in response to pollutant exposure without protein identification. Second are studies that identify these proteins of interest, which can lead to hypotheses of the MoA of the exposure chemical (Tomanek, 2011). The third focuses on post-translational modifications (PTMs).

A number of early studies applied 2DGE to generate unique PES in response to pollutant exposure. When applied to compare zebrafish embryos exposed to either 4-nonylphenol or 17 β -estradiol, differences in the PES's suggest that the MoA of the two chemicals may be more distinct than previously assumed (Shrader et al., 2003).

Taking this comparative approach one step further, SELDI-MS, can be utilized to investigate complex samples

and via a protein chip and MS to explore (still unidentified) expression profiles (Tang et al. 2004). Differences in these mass fingerprints can represent unique signatures that can distinguish different disease states in organisms and are used as biomarkers (Tomanek, 2011). When applied to blue mussels (*Mytilus galloprovincialis*) exposed to oil vs. oil spiked with alkylphenols, it was possible to correctly classify samples from different treatments with 90% accuracy (Bjornstad et al., 2006).

The next level of complexity in proteomic studies is to identify these proteins of interest. In a similar study, juvenile cod (*Gadus morhua*) were exposed to oil or oil spiked with alkyl phenols and polycyclic aromatic hydrocarbons (Bohne-Kjersem et al., 2009). They found that treatment altered protein expression of fibrinolysis, immune function, fertility, calcium homeostasis, fatty acid metabolism, oxidative stress and apoptosis (Bohne-Kjersem et al., 2009).

Unfortunately, estrogenic and androgenic mimics are now common in the environment and detailed proteomic analyses are characterizing their biological effects (Norris and Carr, 2006; Tomanek, 2011). A comprehensive study on the proteomic effects of exposure to the androgenic chemical 17 β -trenbolone, used as a cattle growth

hormone, and its antagonist, flutamide, in livers of fathead minnows (*Pimephales promelas*) showed that a number of proteins functioning in energy metabolism, oxidative and general cellular stress as well as translation changed their abundances (Martyniuk et al., 2009).

The rare minnow (*Gobiocypris rarus*) has been the focus of several toxicological studies. The endocrine disruptor diethylstilbestrol caused several problems in the progeny of exposed *G. rarus*, such as a decrease in testosterone in males, and an increase in estradiol in female fish (Zhong et al., 2005). Another study involving the rare minnow exposed fish to perfluorooctanoic acid, a man-made perfluorinated eight-carbon organic chemical, known to cause liver tumors). The differentially expressed proteins were involved in "intracellular fatty acid transport, oxidative stress, macromolecule catabolism, the cell cycle, maintenance of intracellular Ca^{2+} homeostasis, and mitochondrial function" (Wei et al., 2008).

Proteomics can aid in the assessment of ecosystem health (Tomanek, 2011). Shenandoah River, Virginia, USA, has seen increasing levels of fish death since 2004 (Ripley et al., 2008). Utilizing the resident smallmouth bass (*Micropterus dolomieu*) as an indicator species, this study suggests that the deaths are due to contaminants, because

of changes in metabolism, immune and stress responses investigated via proteomics (Ripley et al., 2008). A similar approach was used with the gold fish (*Carassius auratus*) in the Gaobeidian Lake in Beijing, China (Wang et al., 2008). When fish were exposed to water from either the polluted lake or a reservoir used for drinking water, which contained much lower levels of pollution, this study found changes in the gold fish liver proteome representing energy metabolism (Lb-FABP and betaine homocysteine methyltransferase), anti-oxidative damage proteins (glutathione peroxidase and ferritin) and protein degradation (proteasome).

A study investigating tumors in flatfish (*Limanda limanda*) in the North Sea utilized proteomics and histopathology as indicators for fish health (Stentiford et al., 2005). They found that the "proteome of cancerous liver tissue is different from that of non-tumor liver tissue from the same fish, [and] found similarities between the proteomic changes caused by liver cancer in all of the fish in this study," though these proteins were not identified because SELDI was employed (Stentiford et al., 2005). However, the follow up study found that vitellogenins displayed up to 500-fold induction, in liver tumors (hepatocellular adenoma and carcinoma) in both males

and females. It is likely that "this is the first report of the association of vitellogenin expression with tumors of wild fish," (Small et al., 2010).

The third level of complexity in proteomic studies involves the identification of post translational modifications. While most proteomic studies of fish focusing on PTMs involve development and disease models (see last section), relatively few PTMs are included in fish ecotoxicoproteomic studies. PTMs are an important part of protein processing in the cell, and offer an alternative assessment of the toxic action of pollutants (Tomanek, 2011).

The number of PTMs goes into the hundreds but most pollutant studies focus on carbonylation, glutathionylation, thiol-modifications and ubiquitination (Walsh, 2006). The former three are modifications caused by the increased production of reactive oxygen species (ROS) and can occur due to a change in the oxidative environment of the cell or the activity of specific proteins. In contrast to carbonylation, glutathionylation is a reversible process, which can, under conditions of oxidative stress, protect cysteine residues from oxidation (Sarkar et al., 2006). The main protein degradation pathway in eukaryotic cells utilizes the proteasome, which

digests proteins that are tagged with ubiquitin (Tomanek, 2011). ROSs can cause many modifications which lead to ubiquitination and subsequent denaturation of proteins, including carbonylation of lysine, arginine, proline and threonine; and oxydation of methionine serve as a buffer against a surge in ROS (Dalle-Donne et al., 2003; Levine et al., 1999). Given that a great number of pollutants are known to increase production of ROS, PTMs caused by ROS and subsequent changes in levels of protein degradation, have the potential to be sensitive global markers of pollutant stress.

Focusing on PTMs, a study with medaka (*O. latipes*) investigated the phosphorylation status of fish liver proteins in response to microcystin-LR (Mezhoud et al., 2003). Results confirmed the toxin as a potent inhibitor of protein phosphatase activity via differential phosphorylation patterns found on phenylalanine hydroxylase and keratin. The study focused on phosphorylation because this PTM is "crucial for the selection of metabolic pathways, [therefore toxins can] elicit diverse physiological cell modifications (Mezhoud et al. 2008).

Using systems biology approaches, such as proteomics has been proposed to be an ideal tool to assess effects of pollutants at the cellular level. Even qualitative

descriptions e.g. unidentified PESs, show the systemic changes occurring during exposure are pollutant-specific. Once proteins are identified and categorized by functional group, the mode of action can be hypothesized. Of course, assigning functions should be viewed with some caution since it is by now known that proteins can have multiple functions under different conditions (Tomanek, 2011). Additionally, PTMs present a novel frontier to assess the biological effects of pollutants.

Ecotoxicoproteomics has the potential to become the assessment method of choice for emerging pollutants. These studies can involve acute and chronic exposures as well as exposures to a range of pollutant concentrations that are needed to detect possible non-linear low-dose responses. In order for ecotoxicologists to assess the biological effects of emerging pollutants and to develop comprehensive biomarker profiles, further investigation must be done.

Liver proteomics

C. ios liver tissue was used in this study for several reasons. First, it is one of the largest organs in the fish, which makes it easy to identify and remove, as well as providing enough tissue for analysis. Second, the liver is a major detoxifying organ, and can yield information

about how an organism is dealing with toxic compounds. Third, when doing an exposure experiment, it is likely that different organs and tissues will respond differently which will be seen in their protein expression profiles. Thus, it is important to focus on one organ or tissue, like the liver, to avoid confounding results (Forné et al., 2010). Fourth, fish have important liver features similar to the human liver e.g. both exocrine and metabolic functions occur in the same hepatocyte, a dual blood supply, and a separation of excretory system from hepatocyte function (Di Giulio and Hinton, 2004; Macsween, 1989). These features suggest that fish livers are a good initial model for human liver toxicity investigations. Despite some similarities to mammal livers, fish have what is known as a hepatopancreas. These exocrine pancreatic cells surround the portal veins within the liver can vary in number according to species (Di Giulio and Hinton, 2004).

II. MANUSCRIPT

Abstract

Alkylphenol ethoxylates (APEs) are widely used in industrial and household products as surfactants. APEs degrade into more toxic ethoxylates, such as 4-nonylphenol (NP), which has been shown to be an endocrine disruptor and enhance the growth of tumor cells. Nonylphenol is wider spread in Pacific estuaries than originally thought. Organisms in Morro Bay, California contain some of the highest concentrations of NP reported, while containing few other contaminants. As a benthic mud-dwelling fish, the arrow goby (*Clevelandia ios*) may be exposed to high levels of NP due to high contaminant sequestration rates in anaerobic mud. While ecotoxicology suggests that nonylphenol is in high concentration within *C. ios* tissues along with tissue level biological abnormalities, the molecular effects of nonylphenol on these fish have yet to be investigated. Utilizing proteomic techniques including two-dimensional gel electrophoresis and subsequent identification via MALDI-TOF/TOF mass spectrometry, there is evidence for change in expression of proteins involved in energy metabolism, biotransformation, regulation and cellular structure.

Introduction

Anthropogenic pollution is a worldwide phenomenon and threat to the well-being of many organisms, including our own species. This is in part due to the widespread availability and persistence of what are being described as emerging contaminants, e.g. alkylphenol ethoxylates (APEs) like 4-nonylphenol (Soares et al., 2008). Nonylphenol is a xenoestrogen, a breakdown product of APEs, and commonly used in industrial and household detergents (surfactants), pesticides, and in paper and plastic manufacturing (Ying et al., 2002). NP is known to mimic the hormone 17β -oestradiol, and competes for the oestrogen receptor binding site; has been found to enhance the growth of breast tumor cells and is likely in the environment at levels high enough to cause endocrine disruption (Lee and Lee, 1996; White et al., 1994; Soto et al., 1991; Ying et al., 2002).

Most studies to date have focused on endocrine disruption only; investigating short term exposure, vitellogenin production, and/or histopathology (Arukwe et al., 1997; Bhattacharya et al., 2008;). Those that explore other modes of action (MoA) for the xenobiotic fail to take full advantage of proteomics by not identifying proteins of interest (Stentiford et al., 2005; Shrader et al., 2003).

We chose to focus on the arrow goby (*Clevelandia ios*) because it is a common goby that can be found in estuaries, lagoons, and tidal sloughs along the west coast of North America (Eschmeyer et al., 1983). One estuary, Morro Bay, has been shown to contain high levels of NP in the sediment, with septic systems and waste water treatment plants being the most likely main source (Diehl et al., 2011). In sediment nonylphenol has a half life of 60 years, though under anaerobic conditions, such as those found on the Morro Bay mudflats, it is likely that NP does not undergo any more transformations and remains attached to sludge solids as it partitions favorably into organic materials (Shang et al. 1999, John et al., 2000). We have found that *C. ios* livers contain up to 2700 ng/g wet weight (wet weight [ww]) NP in Morro Bay. Furthermore, we have yet to find an arrow goby population that does not contain nonylphenol. NP's persistent, bioaccumulative, and toxic (PBT) characteristics are of great interest, and it is imperative to determine what effects this chemical exerts beyond endocrine disruption (RIN 2070-ZA09). Utilizing proteomics will allow unbiased insight into how these fish are directly dealing with NP exposure.

We used two dimensional gel electrophoresis (2DGE) and tandem mass spectrometry (MS) to investigate the effects of

NP on *C. ios*. Several recent studies have shown that this approach is successful in generating protein expression data that can generate new hypotheses about the mode of action of environmental stress such as thermal, osmotic, and hypoxic perturbations (Kultz, 1996; Lee et al. 2006; Childress and Seibel, 1998). Protein activity may be the best indicator of survival and fitness (Feder and Walser, 2005). Consequently, investigating changes in protein abundance can tell us how an organism is directly and immediately coping with changes in environment due to stress or environmental toxins, and utilizing 2DGE gives us a snapshot in time of this cellular response.

We have found that NP accumulates within fish tissues quickly over a short period of time. We see differential expression of proteins involved in energy metabolism, biotransformation, regulation and cellular structure.

Materials and Methods

Animal collection and maintenance

Clevelandia ios (Jordan and Gilbert, 1882) were collected November 1, 2009 from the mudflats in Morro Bay, California, USA: 35° 20' 41.28", -120° 50' 39.54". Fish were collected during low tide via slurp guns (a.k.a. yabbie pumps): a hand-pumped low-suction device which removes inhabitants of a water-filled burrow. Species identity was confirmed via visual inspection. Fish were transported via live catch buckets filled with sea water to Cal Poly's Marine Science Research and Education Pier at Avila Beach, California (within 30 minutes). Fish were randomly assigned to one of eight (16 L) sea water tanks equipped with aerators, with a minimum of 33 fish per tank. Tanks were placed in larger temperature-holding tanks, with water from flow-through sea water system keeping a constant temperature in the holding tanks (data loggers measured temperature every 5 minutes, averaged 13.1°C in left block, std dev 0.983; averaged 13.2°C in right block, std dev 1.073 throughout entire experiment). Tank sea water was renewed daily within a three hour window, followed by feeding with fly larvae (Seachem NutriDiet Naturals), which did not contain NP (data not shown).

Experimental design

After a two week acclimation period, with daily water changes and feeding as stated above, 50ppb ($\approx \mu\text{g L}^{-1}$) of NP was added to treatment tanks daily, after water changes and prior to feeding. Control tanks were treated the same as above for the entire experiment, and tanks were randomly assigned to treatment or control. Fish were exposed for sixty days, with fish removed on days 0, 2, 5, 15, 30 and 60 for further analysis. All fish were removed via fishing net and placed in Ziploc bags on dry ice, then moved to -80°C until further processing (Ziploc is produced by SC Johnson, which has released a statement that they are "committed to not use alkylphenol ethoxylate surfactants [in accordance with] EPA's Safer Detergents Stewardship Initiative"). On each collection day fish were collected within a three hour window to avoid any possible differences due to circadian or circatidal rhythms.

Chemical Handling

A fresh 3.68 ppm solution of NP was made up every day (below solubility limit of $4.9 (\pm 0.4) \text{ mg/L}$ in water (Brix et al., 2001), agitated overnight, and diluted into sea water tanks for a final concentration of 50 ppb. NP has a flash point of 141°C , so all equipment was sterilized with

a 5 minute 100% acetone soak, followed by at least two hours at 300°C.

Dissection and Homogenization

Fish for proteomics were dissected on ice, and hepatopancreases were removed and homogenized on ice in 250 μ L homogenization buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 1% ASB-14, 40 mmol l^{-1} Tris-base, 0.5% immobilized pH 4-7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mmol l^{-1} dithiothreitol) using an ice-cold Pellet Pestle homogenizer (Kontes Glass Company, New Jersey, USA). No samples were pooled, and each fish liver represented a single sample. The homogenate was subsequently centrifuged at room temperature for 30 min at 16,100 g and the supernatant was used for further processing. Proteins of the homogenate were precipitated by adding four volumes of ice-cold 10% trichloro-acetic acid in acetone and incubated at -20°C overnight. After centrifugation at 4°C for 15 min at 16,100 g, the precipitate was washed with ice-cold acetone, and centrifuged again before being dissolved in rehydration buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 2% CHAPS, 2% NP-40, 0.002% bromophenol blue, 0.5% IPG buffer and 100 mmol l^{-1} dithioerythritol) through vortexing. The

protein concentration was determined with the 2D Quant kit (GE Healthcare), according to manufacturer's instruction.

Two-dimensional gel electrophoresis

C. ios liver proteins (100 µg) were loaded onto immobilized pH gradient strips (pH 4-7, 11 cm; Bio-Rad) for separation according to their isoelectric point (pI). The isoelectric focusing protocol began with a passive rehydration step (5 h), followed by 12 h of active rehydration at 50V, using an isoelectric focusing cell (BioRad, Hercules, CA, USA). The following protocol was used for the remainder of the run (all voltage changes occurred in rapid mode): 500V for 1 h, 1000V for 1 h, and 8000 V for 5.0 h. The strips were frozen at -80°C. Frozen strips were thawed and incubated in equilibration buffer (375 mmol l⁻¹ Tris-base pH 8.8, 6 mol l⁻¹ urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue) for 15 min each, first with 65 mmol l⁻¹ dithiothreitol; and second, with 135 mmol l⁻¹ iodoacetamide. IPG strips were placed on top of an 11.8% polyacrylamide gel with 0.8% agarose containing Laemmli SDS electrophoresis buffer (25 mmol l⁻¹ Tris-bases, 192 mmol l⁻¹ glycine, 0.1% SDS). Gels were run (Criterion Dodeca; BioRad) at 200 V for 55 min with a recirculating water bath set at 12°C. Gels were subsequently fixed twice for 30

minutes (10% methanol, 7% glacial acetic acid) and stained with SYPRO Ruby fluorescent stain overnight under dark conditions (Biorad). Gels were destained twice for 30 minutes (10% methanol, 7% glacial acetic acid). The resulting gels were scanned with a Typhoon Trio+ fluorescent imager (GE Healthcare).

Gel image analysis

Digitized two-dimensional gel images were analyzed using Delta2D (version 3.6; Decodon, Greifswald, Germany). Group warping strategy was used to connect gel images through match vectors. All images were fused to a composite image or proteome map, which represents average volumes for each spot (Figure 17). Spot boundaries were detected within the proteome map and transferred back to all gel images using match vectors. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image. All automatic software steps were carefully scrutinized and confirmed by eye.

Mass spectrometry

Proteins of interest, i.e. those with a changed expression level due to treatment, were excised from gels using the Bio-rad ProteomeWorks Spot Cutter with Fluorescent Enclosure (cat no. 165-7045), using PDQuest gel

image software (BioRad). Gel plugs were destained twice with 25 mmol l⁻¹ ammonium bicarbonate in 50% acetonitrile, dehydrated with 100% acetonitrile and digested with trypsin (Promega, Madison, WI, USA) overnight at 37°C at a concentration of 11 ng µl⁻¹. Digested proteins were extracted using analyte solution (0.1% trifluoroacetic acid (TFA)/acetonitrile; 2:1), and concentrated using a SpeedVac and rehydrated with 1 µl of analyte solution. The analyte solution was mixed with 5 µl of matrix solution (0.2 mg ml⁻¹ α-hydroxycyano cinnamic acid in acetonitrile) and spotted on an Anchorchip™ target plate (Bruker Daltonics Inc., Billerica, MA, USA), washed with 0.1% TFA and recrystallized using an acetone/ethanol/0.1% TFA (6:3:1) mixture.

Peptide mass fingerprints (PMFs) were obtained on a matrix-assisted laser desorption ionization (MALDI) tandem time-of-flight (TOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc., Billerica, MA, USA). We chose a minimum of twenty peptides to conduct tandem mass spectrometry (time-of-flight) in order to obtain information about the b- and y-ions of the peptide sequence.

To analyze the peptide spectra we used flexAnalysis (version 3.0; Bruker Daltonics Inc.) and applied the TopHat algorithm for baseline subtraction, the Savitzky-Golay

analysis (with: 0.2 m/z; number of cycles = 1) for smoothing and the SNAP algorithm to detect peaks (signal-to-noise ratio: 6 for MS and 1.5 for MS/MS). The charge state of the peptides was assumed to be +1. We used porcine trypsin for internal mass calibration.

To identify proteins we used Mascot (version 2.2; Matrix Science Inc., Boston, MA, USA) and combined PMFs and tandem mass spectra in a search against one main database: MSDB with taxonomy set to Actinopterygii (ray-finned fishes). Oxidation of methionine and carbamidomethylation of cysteine were our only variable modifications. Our search allowed one missed cleavage during trypsin digestion. For tandem mass spectrometry we set the precursor-ion mass tolerance to 0.9 Da. The molecular weight search (MOWSE) score that indicated a significant hit was 36 ($p < 0.05$) in a search against the MSDB Actinopterygii database.

Statistical analysis

Normalized spot volumes were analyzed using an analysis of variance of time x treatment (two-way ANOVA). A null distribution was generated using 1000 permutations and a p-value of 0.01 was used to limit the number of false

positives instead of using a multiple-comparison correction.

Results

Nonylphenol in *C. ios* tissues

Nonylphenol was measured in whole fish tissues (minus digestive tracts, due to potential contamination of ingested sediment) for each day in treatment fish, and at the beginning and end for control fish² (Figure 16).

Figure 16: Concentration of nonylphenol found in whole animals of *C. ios* (GI tract removed) due to laboratory exposure. N=3 for each point, standardized for lipid concentration.

There is a general increase in NP concentration in fish tissues over time (Figure 16). Fish tissue on collection day (-14) contained an average of 80 ppb of NP,

² Due to funding issues, not all samples could be analyzed.

and control fish on day 60 had an average of 214 ppb. NP concentrations are not statistically significantly different for treatment days 0, 2, and 5, and control days -14 and 60 (Figure 16, group a). Treatment days 2, 5, 15, 30 and 60 are not statistically significantly different (Figure 16, group c).

Proteomic analysis

A proteome map showing the average normalized pixel volume of all proteins detected in the 84 analyzed sample gel images was created (Figure 17). After spot boundaries were evaluated within this composite map, 439 individual protein spots were detected. Using an two- way ANOVA ($p < 0.01$ and 1000 permutations), we determined that 28 protein spots (or 6% of the total) showed significant changes in their expression due to treatment, and 13 (or 3%) were time x treatment significant; there is some overlap in these two categories for a total of 37 proteins of interest (8%). Additionally, 122 proteins (28%) changed with time; while these proteins changes may represent tank effect, as our investigation involves NP and NP over time, proteins that change with time only are not identified in this paper.

Figure 17: 2DGE fused image results. 100µg *C. ios* liver tissue, 4–7 pH, stained with SYPRO ruby.

Of the 37 total proteins of interest, 19 spots (or 51%) were successfully identified by MALDI TOF/TOF mass spectrometry (Table 6) in that 1) the score is above the significance threshold and 2) two or more peptide fragments are matched (Pappin et al., 1993). Moreover, three additional proteins were tentatively identified by meeting one of the above mentioned criteria; bringing the ID success up to 59%. Furthermore, while only one collagen was identified, seven proteins within a mass-equal row of spots had peptide mass fingerprint (PMF) patterns almost identical to collagen PMF and thus are likely the same

protein, and suggestive of a post-translational modification pattern. The PMF of these spots had MOWSE scores above the probability threshold set ($p < 0.05$), based on an undisputable similarity with the positively identified collagen, so these were tentatively identified as collagen. If included in the total, this brings our ID success rate up to 76%. Un-identified significant proteins were designated with letters for further differentiation (Figure 17).

Table 6: Protein identifications (using MS/MS) of significant spots. Highlighted cells are interaction-significant, non highlighted cells are treatment-significant (p<0.01, 1000 permutations). Alphabetical order.

Protein ID	Protein abbr.	Spot ID (will be delete)	Theoretical pI/Mw:	Database	GenBank ID	Mowse Score * (/36)	Sequence Coverage (%)	Peptides Matched	MS/MS Sequence Matched	Functional Category
Aldehyde dehydrogenase 6A1	ALDH	A11	8.25 / 57083.14	Matrixsci Msdb actinopt	gi 50539808 NP_001002374.1	162	6	2	K.ENTLNQLVGAAFGAAGQR.C K.AISFVGSNQAGEYIYER.G	Biotransformation
Aldehyde dehydrogenase, putative	ALDH	C3	5.14 / 14053.84	Matrixsci Msdb actinopt	gi 12311783 CAC24485.1	58	15	2	R.GYFIQPTVFQGDVQDNMTIAR.- .- + Oxidation (M)	Biotransformation
Aspartate aminotransferase, cytoplasmic	AST	C7	pI/Mw: 6.53 / 45953.24	Matrixsci Msdb actinopt	gi 47085773 NP_998222.1	48	7	2	K.NFGLYNER.V K.LIVQDDKLNHEYLPILGLPEFR.C	Biotransformation Amino Acid Metabolism
Betaine homocysteine methyltransferase	BMHT	A7	7.56 / 44261.06	Matrixsci Msdb actinopt	gi 190889638 ABF06673.2	45	19	3	K.AGPWTPEATVTHPEAVR.Q R.LNAGEVVGIDGGFVFALEK.R R.AGANVMQITFTFYASDDKLENR. G + Oxidation (M)	Energy Metabolism Biotransformation Amino Acid Metabolism
Collagen, type VI, alpha 1		G2	5.87 / 106609.72	Matrixsci Msdb actinopt	gi 125829720 XP_698253.2	59	2	2	R.VVLVLTGDR.S K.VFAVAISPQEDTR.L	Structure
Cytochrome c oxidase, subunit II	CoxII	C2	4.34 / 31023.64	Marinebak Trembl actinopt	gi 254451555 JZP_05064992.1	41	1	2	R.IAEWPPR.R K.SEPMWHVPASSR.G + Oxidation (M)	Energy Metabolism
Eno3 protein		F8	9.07 / 46662.14	Matrixsci Msdb actinopt	gi 77567762 AAI07495.1	70	9	2	R.AAVPSGASTGVHEALELR.D K.DVILPVPAFNVINGGSHAGNK. L	Energy Metabolism
Fibrinogen beta chain		F5	8.07 / 54372.61	Matrixsci Msdb actinopt	gi 47086505 NP_997939.1	74	7	3	R.GFGNIAFDVGK.G K.EDGGGWYNR.C K.GHCQTPGEYWLGNDR.I + Carbamidomethyl (C)	Structure
Hemopexin-like protein		B10	**	Matrixsci Msdb actinopt	gi 10121689 AAG13324.1	56	13	2	K.VVVGNNHFYR.F K.ELHSDVDVAVFSYDDHLYMIK.D + Oxidation (M)	Oxidative stress
homogentisate 1,2-dioxygenase	HGD	C4	6.20 / 49851.94	Mascot2 EST_actinopt	gi 148298760 NP_694498.2	81/6 1	10	2	K.STRPGVAIADFVIFPPR.W R.GYILEVYGAFELPDLPPIGAN GLANPR.D	Biotransformation Amino Acid Metabolism
Keratin S8, Type I (Oncorhynchus mykiss)		E11	5.28 / 47888.49	Matrixsci Msdb actinopt	gi 185133615 NP_001117826.1	64	3	3	R.NHAEELALR.S R.QSVEADIANLR.R R.QSVEADIANLRR.L	Structure
Keratin S8, Type I (Oncorhynchus mykiss)		E12	5.28 / 47888.49	Matrixsci Msdb actinopt	gi 185133615 NP_001117826.1	57	3	3	R.NHAEELALR.S R.QSVEADIANLR.R R.QSVEADIANLRR.L	Structure
L-xylulose reductase		F9	6.29 / 25824.26	Matrixsci NCBI actinopt	gi 229365856 ACQ57908.1	74	9	2	K.AVLHVSQIVAR.G K.VMALELGPHQIR.V + Oxidation (M)	Biotransformation

Protein ID	Protein abbr.	Spot ID (will be delete)	Theoretical pI/Mw:	Database	GenBank ID	Mowse Score * (/36)	Sequence Coverage (%)	Peptides Matched	MS/MS Sequence Matched	Functional Category
Phenylalanine hydroxylase	PAH	F3	5.86 / 52097.22	Matrixsci Msdb actinopt	gi 74095989 NP_001027850.1	72	6	3	R.DFLAGLAFR.V R.FLQSGTGFR.L + Carbamidomethyl (C) K.EFADIAYNR.H	Biotransformation Amino acid metabolism
Phenylalanine hydroxylase	PAH	C1	5.86 / 52097.22	Matrixsci Msdb actinopt	gi 74095989 NP_001027850.1	47	4	2	R.DFLAGLAFR.V R.LRPVAGLLSSR.D Amino acid and protein	Biotransformation Amino acid metabolism
Phosphoglucosutase-1	PGM	A10	5.74 / 61090.00	Matrixsci Msdb actinopt	gi 41056111 NP_957319.1	87	8	3	R.GIFDFAALK.Q K.LSLCGEESFGTGS DHIR.E + Carbamidomethyl (C) K.IITVKTTPYADQKPGTSGLR.K	Energy metabolism
Ubiquinol-cytochrome c reductase core protein 2	Uqcrc 2	A12	7.72 / 47787.31	Matrixsci Msdb actinopt	gi 197632233 ACH70840.1	124	5	2	K.QVGEQFLNIR.S R.MALVGLGVDHTVLK.Q + Oxidation (M)	Energy metabolism
Wnt 8.2		E5	9.20/390 67.16	Matrixsci Msdb actinopt	gi 49659797 AAT68195.1	41	2	2	R.LCQDCGLR.V R.RLCQDCGLR.V	Signal transduction
Wnt-10a		E6	8.71 / 50014.48	Matrixsci swissprot actinopt	gi 1175018 P43446.1	35/2 5	4	2	K.ACGCDEKR.R + R.DLGSDSAGTQGR.I	Signal transduction
(cystic fibrosis transmembrane conductance regulator)***	CFTR	E2	8.59 / 168291.7 7	Matrixsci Msdb actinopt	gi 113678252 NP_001038348.1	35/3 6	1	2	K.ACQLEEDLAALPEK.D + Carbamidomethyl R.QSVLAFMTNAQQQGR.R	Biotransformation
(Estrogen receptor)	ER	F6	8.23/633 21.10	Mascot2 SwissProt Actinopt	gi 5596999 CAB51479.1	24/2 2	1	1	R.GAEPMLCSR.Q Carbamidomethyl (C)	Energy Metabolism Regulation
(Warm temperature acclimation related-like 65kDa protein)	HSP	E10	5.31 / 48371.55	EST library	gi 169807830 CAL59578.1	113/ 61	7	1	K.ELDDIHGIGHVDAAFR.M	Oxidative Stress
<p>*MOWSE score out of 36, unless otherwise stated.</p> <p>**Sequence contains several consecutive undefined AA. Its pI and Mw cannot be computed.</p> <p>***Proteins in parentheses were identified with only one matched peptide, or are just under significance score.</p> <p>Highlighted cells are Interaction significant, non highlighted cells are Treatment significant</p>										

Hierarchical clustering organizes significantly changing proteins based on similar expression pattern, and we have separated these into interaction or treatment significant categories (Figure 18).

Figure 18: Heat map of all significant and identified proteins. Columns are individual fish, broken into groups: C = control, NP = nonylphenol exposed. Rows are individual proteins. Blue represents down-regulation, red is up-regulation. Figure includes interaction and treatment proteins separated, and organized through hierarchical clustering.

Proteins by functional category

Another way to visualize these results is to group proteins into categories based on their function. Because of the complexity of data in this experiment, there is no "fold change" category in the list of changing proteins

(Table 6), and comparing heat map clusters may be difficult to interpret (Figure 18). Individual expression has been tracked comparing treatment (50 ppb NP) and control specimens for each sampling date, yielding proteins that are treatment or interaction (time x treatment, i.e. proteins that change with treatment over time) significant.

Proteins that are changing due to nonylphenol exposure are organized into functional categories: energy metabolism, biotransformation (including oxidative stress, amino acid metabolism, and structure) and regulation.

There are several changing proteins which are involved in energy metabolism (Table 7). Phosphoglucosmutase-1 (PGM) is interaction-significant; this protein is involved in glycolysis or the pentose phosphate pathway, and is up-regulated with NP during each day except day 2, though with similar expression between control and treatment on day 60. A treatment-significant protein is eno3, or β -enolase, which is down-regulated with NP exposure and is the enzyme catalyzing 2-phosphoglycerate to phosphoenolpyruvate in glycolysis, and proceeds via metal ion catalysis (Lehninger et al, 2004).

Two electron transport chain proteins, ubiquinol-cytochrome c reductase core protein 2 (Uqcrc2) and cytochrome C oxidase subunit 2 (CoxII), are both down-

regulated with NP treatment. These proteins are both members of the electron transport chain (ETC). Uqcrc2 is interaction significant and down-regulated with NP exposure in all days after day zero. This protein is a member of ETC Complex III, also known as cytochrome bc₁ complex or ubiquinone:cytochrome c oxidoreductase (Lehninger et al., 2004).

Table 7: Protein expression profiles of significant proteins involved in Energy Metabolism

Cytochrome oxidase is the final electron acceptor in the ETC, called complex IV or cytochrome oxidase, which takes electrons from cytochrome c, and reduces oxygen to water (Lehninger et al., 2004). This is a large 13 subunit complex and subunit II is essential for function in that it contains copper which is the primary acceptor of electrons from the reduced cytochrome C (Ostermeier, 1997).

Next, several proteins are involved in biotransformation and detoxification (Table 8). Two isoforms of aldehyde dehydrogenase (ALDH) were identified. ALDH 6A1 was up-regulated with treatment on days 0, 2 and 15, while down-regulated on days 5, 30 and 60. Putative ALDH is generally up-regulated in NP (though slightly down only on day 30). ALDH family enzymes oxidize aldehydes to carboxylic acids, 6A1 is a mitochondrial ALDH which is CoA-dependent and involved in the catabolism of valine and pyrimidine (Alnouti & Klaassen, 2008).

L-xylulose reductase increased with NP exposure in the beginning, days 0, 2, 5, and 15, but decreased on days 30 and 60. This protein is involved in NADH-dependent L-arabinose catabolism, converting L-xylulose to xylitol (Verho et al., 2004). Hemopexin is up-regulated with NP exposure, and is a heme-binding scavenger of reactive oxygen species (Piccard et al., 2007).

Table 8: Protein expression profiles of significant proteins involved in Biotransformation

The heat shock protein (HSP) Warm-temperature-acclimation-related-65 is increased with NP exposure though

was only tentatively identified (significant MOWSE score, only one matched MS/MS peptide, Table 6).

Next, several significant proteins are involved in amino acid metabolism (Table 9), which may also be involved in biotransformation. Betaine homocysteine methyltransferase is an interaction-significant protein, up-regulated on day 2, down on day 5, up again for days 15 and 30, and finally down-regulated on day 60. This enzyme is a single carbon transferase, catalyzing betane and homocysteine into dimethylglycerine and methionine (Purohit et al., 2007).

Two isoforms of phenylalanine hydroxylase (PAH) were identified, one was interaction significant; down-regulated at the beginning and end of the experiment: down in days 0, 2, and 5, up-regulated on days 15 and 30, then down again on day 60. The second PAH is treatment significant, and is down-regulated in NP exposure. This protein is involved in the first and rate-limiting enzyme degradation pathway for conversion of phenylalanine to tyrosine, and is a mixed-function oxidase (Epperson et al., 2004; Lehninger et al., 2004).

Table 9: Protein expression profiles of significant proteins involved in Amino Acid Metabolism

Additionally, aspartate aminotransferase (AST) is down-regulated with NP. This enzyme is responsible for the

conversion of oxaloacetate and glutamate into aspartate and α -ketoglutarate, and its increase in the bloodstream can be important for the diagnosis of liver damage and toxicity (Lehninger et al., 2004). Homogentisate 1,2 dioxygenase expression is complicated, with down-regulation on days 0, 2 and 30, and up-regulation on days 5, 15, and 60. This protein is involved in cleaving the aromatic ring in metabolism of phenylalanine and tyrosine (Titus et al., 2000).

Next, several proteins involved in structure were significant (Table 10). The tentatively identified cystic fibrosis transmembrane conductance factor was up-regulated on day zero with NP, down- on days 2 and 5, and up-regulated with treatment on days 15, 30 and 60 (MOWSE 35/36 score, with two matched MS/MS peptides, Table 6). This protein is an epithelial cell membranes ABC transporter used to move chloride and thiocyanate (Childers et al., 2007).

Collagen (type VI, alpha 1) is down-regulated with NP exposure and is an extracellular matrix protein. Though seven total proteins belonging to a row of post-translationally modified spots were significantly decreased with NP exposure (Figure 17, see upper right corner), only

one was positively identified as collagen (although others had close to identical PMFs).

Table 10: Protein expression profiles of significant proteins involved in Structure

Fibrinogen beta polypeptide is up-regulated with NP exposure, and is involved in blood-clotting, and is converted into fibrin (via thrombin) to make a clot (Lehninger et al., 2004). Two isoforms of keratin S8 Type I were significant, both were identified as a fish keratin (*O. mykiss*), an acidic (type I) intermediate filament. One keratin was down-regulated with NP exposure, while the second was interaction-significant and had similar expression in control and treatment fish over days 0 and 5, down-regulation with treatment on days 2, 15, and 30, and up-regulation on day 60.

Finally, several proteins involved in regulation were identified (Table 11). Two Wnt proteins were significant; Wnt 8.2 is down-regulated during each day except day 30; Wnt 10a is down-regulated during each day except day 60. Wnt family proteins are involved in signal transduction and proteolysis (Lodish et al., 2004). The tentatively identified estrogen receptor (significant MOWSE score, only one matched MS/MS peptide, Table 6) is down-regulated with NP exposure.

Table 11: Protein expression profiles of significant proteins involved in Regulation

Highlighted graphs are interaction significant proteins.

Discussion

Nonylphenol in *C. ios* tissues

Looking at NP in exposed fish tissue (Figure 16); exposed fish tissues have a significantly increased level of NP c.f. control fish. And while not statistically significant, there is a general trend of increase in NP concentration in exposed fish over time. Since there are few long-term studies it is not possible to compare our data with others.

Energy Metabolism

There seems to be a systematic shutdown of general energy metabolism (Table 7), though Phosphoglucomutase-1 (PGM) is generally up-regulated with NP exposure and catalyzes glucose 1-phosphate into glucose 6-phosphate (G6P). G6P can then enter either glycolysis or the pentose phosphate pathway. One product of the pentose phosphate pathway is NADPH, which can be used to counter the effects of reactive oxygen species, many of which are often generated through phase I of biotransformation (Lehninger et al., 2004; Di Giulio and Hinton, 2008). Tanguy et al. (2006) also saw an increase in PGM mRNA expression in response to xenobiotic exposure in *C. gigas*.

A treatment significant, metabolism protein that is down-regulated with NP exposure is enolase, which catalyzes 2-phosphoglycerate into phosphoenolpyruvate during glycolysis. Terasaka et al. (2004) also saw a decrease in Eno3 production by MCF-7 cells exposed to NP and other industrial chemicals.

Two treatment significant proteins involved in the electron transport chain are ubiquinol-cytochrome c reductase core protein 2 (uqcrc2) and cytochrome C oxidase subunit 2 (CoxII), which are both down-regulated in NP treatment. Uqcrc2 is a member of ETC complex III, also known as cytochrome bc₁ complex or ubiquinone:cytochrome c oxidoreductase (Lehninger et al., 2004). This complex couples electron transfer from ubiquinol to cytochrome C with the pumping of protons from the matrix to the inner membrane space in the mitochondria. The "core" proteins - which are located on the periphery- are members of the mitochondrial processing peptidase (MPP) family of proteins, which might be involved in importing proteins into the mitochondrion for further processing (Iwata et al., 1998).

Cytochrome oxidase is the final stage in the ETC, called complex IV or cytochrome oxidase, which takes electrons from cytochrome C, and reduces oxygen to water

(Lehninger et al., 2004). This is a large 13 sub-unit complex, and subunit II is essential for function, containing the copper which is the primary acceptor of electrons from the reduced cytochrome C (Ostermeier, 1997).

Two biological states can lead to a suppression of metabolic activity. First, in response to stress, the body will release excess cortisol, decreasing thyroid hormone, and reducing the uptake of glucose (Ripley et al., 2008; Vijayan et al., 1991). Other environmental toxicants, such as arsenic and the xenobiotic pesticide malathion, have been found to decrease respiratory burst activity (Hermann and Kim, 2005; Beaman et al., 1999).

Second, endocrine disruption may also lead to a metabolic disruption, i.e. decrease in metabolism (Casals-Casas and Desvergne, 2011). We see that NP exposure leads to a decrease in ER, which may be due to a negative feedback loop due to endocrine disruption. ER knockout mice showed a reduced glucose tolerance, and a higher level of white adipose tissue (Heine et al., 2000).

Biotransformation

One of the main functions of the liver is the biotransformation (or metabolism) of lipophilic, including xenobiotic compounds (Di Giulio and Hinton,

2008). In order to metabolize and eventually excrete nonpolar lipophilic xenobiotics, like nonylphenol, a several step biotransformation of the compound may occur in order to make the compound more water-soluble. Phase I includes oxidation, reduction or hydrolysis of the compound to enhance polarity (Di Giulio and Hinton, 2008). Phase II will involves enhancing the polarity further, shielding functional groups, or conjugation with amino acids. Phase III involves elimination from the cell.

Phase I oxidation enzymes include dehydrogenases, which transfer electrons to a cofactor, like NAD^+ . We have a putative aldehyde dehydrogenase (ALDH), as well as aldehyde dehydrogenase 6A1 as an example. This enzyme has a complex expression pattern in treatment and interaction (respectively). The first is generally up-regulated, while ALDH 6A1 is down-regulated with long term (after d 30) exposure.

ALDH's are known xenobiotic metabolizers, involved in the oxidation of aldehydes to carboxylic acids. Furthermore, a bacterial alcohol dehydrogenase (ADH) has been found to degrade short chain nonylphenol (Hjelle et al., 1983; Alnouti & Klaassen, 2008; Liu et al., 2007). ADH is also a phase I enzyme, oxidizing alcohols to aldehydes, which are further converted to acids by ALDH.

ALDH 6A1 is involved in valine catabolism (Alnouti & Klaassen, 2008). Furthermore, ALDH's are involved in beta oxidation, which is important in fatty acid catabolism to make energy and more water soluble molecules (Lehninger et al., 2004). Additionally, Tanghe et al. (1999) suggest that the breakdown of NP may begin with the breakdown of the alkyl chain, thus giving further support to the potential role of ALDH in the metabolic breakdown of NP.

Reductases are important phase I biotransformation enzymes. We have found L-xylulose reductase to be increased with NP exposure at the beginning of the experiment, and decrease after day 30. L-xylulose reductase has broad substrate specificity, and is thought to be involved in the metabolism of xenobiotic compounds (Matsunaga et al., 2006).

Since these three phase I detoxifying enzymes all have similar expression changes around day 30, it is possible that there is a shift in how gobies process NP after this time. Furthermore, the abovementioned proteins are next to each other on the heat map, showing similar expression patterns (Figure 18).

Unfortunately, while phase I enzymes are enhancing polarity of toxic chemicals, they can also create reactive oxygen species (ROS), which are extremely hazardous to the

cell, and indicative of stress (Hinton). It is possible that this is why we see up-regulation of hemopexin with NP exposure, a ROS scavenger (Piccard et al., 2007). This might also explain why we see an increase in Warm-temperature-acclimation-related-65, a general heat shock protein, with NP exposure (Smith et al., 2011).

Phase II biotransformation enzymes either enhance the polarity further than phase I, add groups functional groups to(i.e. methyl, acetyl) supply steric hindrance, or can add amino acids (amino acid conjugation) to enhance the pH for more efficient excretion (Di Giulio and Hinton, 2008). While taurine is the amino acid that has been studied most for these purposes (James, 1987), we have four enzymes that are involved in amino acid metabolism: betaine homocysteine methyltransferase, two isoforms of phenylalanine hydroxylase, aspartate aminotransferase, and homogentisate 1,2 dioxygenase.

Betaine homocysteine methyltransferase was generally up-regulated with NP until day 30, and down-regulated on day 60. This enzyme is important in regulating intracellular methionine levels and subsequent methylation; working as a single carbon transferase, catalyzing betaine and homocysteine into dimethylglycerine and methionine, methionine can be further degraded into succinyl-CoA, a

citric acid cycle intermediate (Martyniuk et al., 2009; Lehninger et al., 2004). This also helps support the down-regulation of energy metabolism we see, especially after day 30. Additionally, taurine is a down-stream product of this enzyme, which may be conjugating with NP for biotransformation purposes. This protein is regulated by the androgen receptor as and as NP is an anti-androgen, BHMT may be important for future biomarkers of environmental contamination (Lee et al., 2003; Martyniuk et al., 2009). Wang et al. (2008) also saw a decrease in BHMT in fish livers exposed to contaminated lake water.

Figure 19: Catabolic pathways for phenylalanine and tyrosine. (Fig 18.23 from Lehninger et al., 2004).

Next, we see changes in three enzymes involved in phenylalanine and tyrosine catabolism (Figure 19; Lehninger et al., 2004).

Treatment significant phenylalanine hydroxylase (PAH) is down-regulated in NP exposure, and is the first and rate-limiting step in the pathway, as well as a mixed-function oxidase (also known as phenylalanine 4-monooxygenase; Epperson et al., 2004). Because NP and PAH have similar structures (see Table 4 and Figure 19), it is possible that down-regulation is due to a negative feedback

cycle from NP exposure. Though this could be a mechanism to maintain an amino acid pool for protein reconstruction; however PAH has been indicated in biotransformation of other xenobiotics (Epperson et al, 2004; Steventon et al., 2009). Acevedo et al. (2005) also saw a decrease in PAH with NP exposure for 32 weeks on mice, but do not provide any possible explanation.

Aspartate aminotransferase (AST) is also down-regulated during NP exposure, is responsible for the conversion of oxaloacetate and glutamate into aspartate and α -ketoglutarate (Lehninger et al., 2004). The direction of this reaction is reliant on a pyridoxal phosphate cofactor to transfer the amino group in the appropriate direction (Kirsch et al., 1984). In one direction, AST's products are the reactants of PAH, and it is possible that there is a futile cycle here. Though, working in the opposite direction might reinforce the down-regulation of this pathway, and a down-regulation of oxaloacetate again supports our hypothesis of a down-regulated metabolism. Bhattacharya et al. (2008) also saw a decrease of AST activity with NP treatment of fish liver.

Homogentisate 1,2 dioxygenase expression is a bit more complicated, with down-regulation on days 0 and 2, up-regulation on days 5 and 15, down again on day 30, and up

on day 60. This protein is involved cleaving the aromatic ring in metabolism of phenylalanine and tyrosine (Titus et al., 2000). End products of this pathway include fumarate, which is important to the citric acid cycle.

Catabolism of each of the above discussed amino acids can feed into the citric acid cycle (Figure 20). Since almost all of these are down-regulated by day 60 (Table 9), this further supports our hypothesis of a general down-regulation of energy metabolism.

Figure 20: Summary of amino acid catabolism (Fig 18.15 from Lehninger et al., 2004)

Though traditionally only including two phases, in 1992 Ishikawa proposed a phase III of biotransformation, which included eliminating metabolites from the cell, specifically using ATP-dependent export pumps. Cystic fibrosis transmembrane conductance factor (CFTR) is an ABC-transporter that use ATP to transport chloride and thiocyanate ions; and can also transport glutathione (Gadsby et al., 2006; Childers et al., 2007). CFTR is mostly up-regulated in NP, c.f. control fish

Furthermore, in order to excrete xenobiotics from the cell, a cellular rearrangement would have to occur. We see several structural proteins changing including two keratins and seven type VI collagens. Both keratins were generally down-regulated with exposure and were confirmed as fish (*O. mykiss*) type I Keratin S8, rather than potential human contamination.

Collagen (type VI, alpha 1) is down-regulated with NP exposure and is an extracellular matrix protein. Seven total proteins in this potential PTM pattern were significantly decreased with NP exposure, and one was positively identified as collagen (type VI, alpha 1). The other six proteins were tentatively identified as collagen as well, with nearly identical peptide mass fingerprints to

the identified collagen. These proteins had a similar down-regulation as can be seen on the heat map (Figure 18). Collagen VI has many roles in the cell, including maintaining tissue integrity. Degradation of this collagen can lead to shedding of epithelial cells from the basement membrane; while a deficiency can lead to apoptosis (Lemjabbar et al., 1999; Irwin et al., 2003).

Matrix metalloproteinases/gelatinases contain a hemopexin-like domain, as we have a significantly up-regulated hemopexin like protein, this could help to explain the collagen activity (Novotna et al., 2002).

Fibrinogen beta polypeptide is up-regulated with NP exposure, and is involved in blood-clotting, and is converted into fibrin (via thrombin) to make a clot (Lehninger et al., 2004). Williams et al. (2007) also saw an up-regulation of fibrinogen with 17 β -estradiol exposure in fish, and suggest this is a proliferation protein. Luyendyk et al. (2011) found an increase in all three fibrinogen peptides (α , β , γ) as well as plasma fibrinogen with addition of a chemical liver injury-inducer.

Next, two Wnt proteins were significant with NP exposure. Wnt family proteins are involved in signal transduction and proteolysis (Lodish et al., 2004). Wnt 8.2 is down-regulated during each day except day 30; Wnt

10a is down-regulated during each day except day 60. Martyniuk et al. (2007) saw a decrease in Wnt 10a with exposure to 17alpha-ethinylestradiol.

Collagen and fibrinogen are mediated by extracellular integrins, and changes to these proteins are involved in metastasis and cancer (Cairns et al., 2003). Additionally, Wnts can regulate collagen and other extracellular matrix metalloproteinases (Schambony et al., 2004). These Wnts and collagens are also next to each other on the heat map (Figure 18), the hierarchical clustering suggests that these proteins have a similar expression pattern, and we may infer that they are involved. Taken together, this might indicate that there is massive tissue remodeling happening due to NP exposure.

Finally, we have a tentatively identified estrogen receptor, which is down-regulated with NP exposure. It is possible that since NP is an estrogen mimic, that a negative feedback cycle is leading to a decreased level of estrogen receptors. Additionally, estrogen receptors are known to regulate energy metabolism, this could be why we see a decrease in over-all metabolic proteins (Giguere, 2008; Roper et al., 2008; Chen et al., 2009).

Again, two biological states can lead to a suppression of metabolic activity. First, in response to stress, the

body will release excess cortisol, decreasing thyroid hormone, and reducing the uptake of glucose (Ripley et al., 2008; Vijayan et al., 1991). Other environmental toxicants, such as arsenic and the xenobiotic pesticide malathion, have been found to decrease respiratory burst activity (Hermann and Kim, 2005; Beaman et al., 1999).

Second, endocrine disruption may also lead to a metabolic disruption, i.e. decrease in metabolism (Casals-Casas and Desvergne, 2010). We see that NP exposure leads to a decrease in ER, which may be due to a negative feedback loop due to endocrine disruption. ER knockout mice showed a reduced glucose tolerance, and a higher level of white adipose tissue (Heine et al., 2000). Ultimately, endocrine disrupting compounds are being investigated as a cause for the worldwide epidemic of obesity. Furthermore, oral contraceptives, which contain synthetic estrogens and progesterones, are known to impair glucose tolerance (Vijayalakshmi et al., 1988). Our data supports this theory, with a less efficient energy metabolism leading to a higher degree of fat deposition. Furthermore, this may be a physiological defense, as there is a negative correlation between BMI and NP concentration, and toxins stored in adipose tissue (including lipid soluble ones, like NP), often mobilize with weight loss (Lopez-Espinosa

et al., 2009; Liska, 1998). If this is the case, nonylphenol exposure is extremely toxic, by decreasing energy metabolism.

Figure 21: Summary of cellular effects of nonylphenol. Blue represents down-regulation, red is up-regulation, green is other. Pink rectangles are amino acids undergoing catabolism.

In summary (Figure 21), we see proteins changing due to NP exposure that are involved in energy metabolism, with a down-regulation of proteins involved in glycolysis and the electron transport chain. The pentose-phosphate pathway may be up-regulated to increase levels of NADPH to

remove ROS. Additionally, ROS can be removed via hemopexin, and we see an increase in HSPs to aid in this and protein mis-folding due to cellular stress. Catabolism of amino acids may also be contributing. A shift in structure is seen in changing keratins; as is a major down-regulation of extracellular collagens, which may be regulated by Wnts (Figure 21).

Figure 22: Summary of the effects of nonylphenol.

Overall (Figure 22), we see that proteins changing with NP exposure are involved in energy metabolism, biotransformation (including phase I and ROS scavengers, phase II and amino acid metabolism, phase III and structure), and regulation. We also see that NP increases

in fish tissues over time, and treatment fish had a statistically significantly different amount of NP in their tissues, as compared with control fish. We have also seen that NP is a widely used surfactant, and is very persistent and prevalent in the environment.

Implications

Our inability to discover a single population of *C. ios* without contamination underscores the prevalence of NP in our environment. This chemical has sparked global interest; the European Union has restricted the use and marketing of nonylphenol and its ethoxylates (Directive 2003/53/EC). Canada has banned the use of NPEs in detergents, and aquatic safety limits are set to 1.0 ppb in freshwater and 0.7 ppb in seawater (CWQGPAL, 2002).

The USEPA has recently developed an action plan for NP and NPEs including the voluntary phase-out of NPEs in industry and rule making under the Toxic Substances Act (RIN 2070-ZA09). The demand in the United States for NP was estimated to be 380 million pounds in 2010 (RIN 2070-ZA09). Though yet to be regulated, the EPA's Office of Water has recommended water quality criteria (WQC) for NP. The WQC for freshwater species is 28 ppb acute and 6.6 ppb

chronic exposure, and for saltwater species at 7 ppb acute and 1.7 ppb chronic exposure (RIN 2070-ZA09).

Nonylphenol and its ethoxylates are amphipathic, with a hydrophobic head (the alkylphenyl moiety) and a hydrophilic tail (the ethoxylate chain). These characteristics make NP a successful industrial surfactant as well as cause nonylphenol to be persistent in the environment where it may bioaccumulate through the food chain (Diehl et al., 2011).

Alternatives to nonylphenol ethoxylates include other APEs, which can still be used as emulsifiers, dispersants, and surfactants (RIN 2070-ZA09). These include "glucose-based carbohydrate derivatives such as alkylpolyglucoside, glucamides, and glucamine oxides," (RIN 2070-ZA09). Other alternatives are alcohol ethoxylates, which are less efficient in industry, but degrade faster and therefore may be safer for the environment (Soares et al., 2008). Unfortunately these alternatives have not been around long enough to research the potential effects of breakdown products on organisms (Soares et al., 2008).

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