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LITERATURE SURVEY:

EFFECTS OF HYDROCARBONS ON MUSSEL GENOMICS

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Abstract

Industrial activities in coastal areas throughout the world have heightened concerns over degradation of marine environments, including contamination resulting from oil and gas development. Petroleum hydrocarbons have long been recognized to have genotoxic effects, with exposures leading to a range of effects including mutations and cancer. Effects of petroleum exposure have been extensively studied for decades; however, in recent years there have been major advances in new technologies for elucidating the genome and measuring alterations in expression of specific genes in response to contaminant exposure. Herein we provide a review of the literature on developing genomic technologies for the detection of genotoxic effects in mussels and other bivalve species, with a focus on effects of petroleum hydrocarbons. This review was compiled at the request of the Prince William Sound Regional Citizens Advisory Committee. In addition to this review document, we have assembled an annotated list of relevant references including those within this document and additional ones that are not cited here. These references are provided in a separate format (excel file).

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Introduction

Background and Need

Threats to coastal marine areas arise from multiple sources, including pollution, invasive species, over-exploitation, loss of biodiversity, and climate change (Harley et al. 2006, Lotze et al. 2006, Crain et al. 2009, Doney et al. 2012, Maslo and Lockwood 2014). Environmental contaminants are a major concern and will continue to pose a risk with increasing occupation and development of coastal areas. Some contaminants result from local sources of pollution, whereas others are transported globally. Broad classes of contaminants of concern in coastal marine environments include persistent organic pollutants (including polycyclic aromatic hydrocarbons, polyhalogenated biphenyls, brominated flame retardants, organometals, and pharmaceuticals), heavy metals, and plastics (Crain et al. 2009, Cole et al. 2011, Gaw et al. 2014).

Arctic and subarctic coastal and offshore areas face pressure from industrial development, particularly for production and transport of oil and gas, activities that will accelerate as sea ice diminishes in a warming climate. Industrial development leads to increased risk of petroleum and other contaminants in the nearshore environment, from a variety of sources. The 1989 *Exxon Valdez* oil spill (EVOS) demonstrated widespread and relatively long-lasting consequences of a large release of crude oil. Studies of biological resources in the aftermath of the spill documented lethal effects of acute oil exposure, as well as long-term injury to individuals, populations, and ecosystems from exposure to residual oil (Rice et al. 2001, Peterson et al. 2001, Rice 2009, Bodkin et al. 2014, Ballachey et al. 2014, Shigenaka 2014).

Hydrocarbon contaminants released into the marine environment from non-point sources (e.g., atmospheric deposition, surface runoff) far outweigh the amount released from marine vessel spills (NRC 2003) and thus present an ongoing concern for the health of marine ecosystems (Law and Klungsoyr 2000, Peterson et al. 2003, Hylland 2006, Bodkin et al. 2014). However, the extent and duration of effects from chronic exposure to low levels of hydrocarbons can be difficult to address. Further, the presence of multiple stressors including mixtures of environmental contaminants, increasing temperature, and decreasing pH, raises the potential for harmful cumulative impacts and interactions and impedes our ability to understand underlying causes and mechanisms of long-term effects (Adams 2005, Crain et al. 2008, 2009, Holmstrup et al. 2010, Whitehead 2013, Coelho et al. 2015).

Given mounting pressures on the environment, efficient and effective approaches to monitor health of ecosystems are needed. For several decades, mussels (*Mytilus* spp.) filter feeding bivalves commonly found in intertidal areas, have been used as a bioindicator species in coastal marine monitoring programs, and numerous measures have been developed to assess mussel health and status. Herein we provide a brief review of methods that have been used to evaluate genotoxic effects (i.e., damage to the DNA molecules) of petroleum contaminants in mussels, and describe advances in genomic technologies that have promise in research and monitoring programs and are applicable to mussels and other species.

To accomplish this review, we conducted word searches for general terms related to the confluence of mussel, hydrocarbon, and genomic bodies of literature (keyword examples: mussel, *Mytilus*, bivalve, hydrocarbon, genomics, transcription, next generation sequencing). We did not restrict publication dates; however, dates were somewhat self-restricting due to the relatively recent emergence of genomics technology, which was our focus. Inclusion criteria encompassed research on mussels under controlled laboratory conditions and in field studies, written in English, with primary content related to the effects of hydrocarbons on genomics. Where relevant, studies on other bivalve species were included. Following the initial full-text review, additional sources were added through forward citation searching to ensure thorough coverage of available literature. The final analysis included citations that were included in the review document and several hundred additional citations which were not cited in the review document but which we felt were important to a broader background understanding of the techniques. All citations and their abstracts are incorporated in an excel file which accompanies the review document. The citations included in the excel file were organized into categories: fish, invertebrates, micro-organisms, mammals, general, and emerging technologies/RNA sequencing.

Polycyclic Aromatic Hydrocarbons

Crude oil is a mixture of many different hydrocarbons, including multiple aromatic and aliphatic compounds. The composition of crude oil is highly complex and variable, and is unique to the source of the oil. One group of compounds from crude oil is polycyclic aromatic hydrocarbons (PAHs), formed of carbon and hydrogen and characterized by the presence of two or more fused aromatic rings (Srogi 2007, Pampanin and Sydnes 2013). PAHs are ubiquitous in the environment, arising primarily from the combustion of or contamination of petroleum-based products used as energy sources for extant civilizations.

Effects of exposure to PAHs have been extensively studied for many decades due to their widespread presence in the environment and recognition that exposure can have serious consequences for biota (Suess 1976, Eisler 1987, Meador et al. 1995, Mastrangelo et al. 1996, Douben 2003, Peterson et al. 2003, Hylland 2006, Reynaud and Deschaux 2006, Srogi 2007, Billiard et al. 2008, Whitehead 2013). Specific effects of exposure can vary greatly, influenced by the conditions of the exposure (e.g., concentrations, route, duration), specific PAH compounds, presence of other stressors (e.g., disease, competition, climate factors), the species affected and stage of development at the time of exposure. Laboratory studies of PAH effects can be highly useful as conditions can be controlled, but generally do not reflect the complexities of environmental conditions and stressors experienced by organisms in natural systems.

Research on exposure to petroleum-based hydrocarbons conducted over several decades following EVOS demonstrated a broad range of consequences, and highlighted the importance of both acute and chronic effects. Early studies of EVOS effects on biota (mammals, birds, fish, invertebrates, and microbial communities) were summarized in a series of manuscripts compiled by Rice et al. (1996); continuing research findings with a longer-term perspective have been presented by Peterson et al. (2003), Carls and Meador (2009), Rice (2009), Miles et al. (2012),

Wiens (2013), Bodkin et al. (2014), Ballachey et al. (2014), Fukuyama et al. (2014), and Shigenaka (2014). An important overall finding from the collected body of EVOS research is that an understanding of chronic, frequently subtle, effects of PAH exposure is critical for predicting long-term effects on populations and, accordingly, ecosystems.

Biomarkers for Monitoring PAH and Other Contaminants

Environmental assessment and toxicology studies historically have relied on occurrence or burden of foreign compounds (xenobiotics) in tissues of various species as an indicator of toxic insult. However, compounds in tissues may be metabolized and excreted, thus analyses of contaminant burden may not provide insight into the welfare of the individual. Further, body burdens can be affected by multiple factors including type and concentration of contaminant, period of exposure, species, metabolic processes, condition of individual, stage of development, and body tissue assayed. Other metrics are needed to monitor exposure and effects of the exposure.

Over the past decades, numerous biomarkers have been developed to assess exposure to contaminants and potentially, resulting effects on individual health. Biomarkers can be defined as “molecular and/or cellular alterations that occur along the temporal and mechanistic pathways connecting ambient exposure to a toxicant and eventual disease (DeCaprio 1997), and typically, can be classified as indicators of exposure, effect, or susceptibility (DeCaprio 1997, Silins and Högberg 2011). Defined more broadly, biomarkers can be considered as measures of responses to both chemical and physiological stressors, and are not limited to responses at the cellular level but can include changes in growth, reproduction, or behavior as well. Development and application of biomarker technologies have been driven by the need for a more comprehensive approach to environmental and ecotoxicological assessment, beyond physical and chemical measurements (Depledge 1998, Peterson et al. 2003).

A large number of biomarkers have been developed and evaluated over the last several decades. The utility of a biomarker will depend on a number of criteria, including chemical specificity, dose-response and time-response relationships, sensitivity, and feasibility of measurement. Biomarkers that have been widely used include enzymes and proteins, metabolites of xenobiotic chemicals, histopathological changes, immunological changes, and DNA alterations. Huggett et al. (1992) compiled an extensive early review of the types and utility of different biomarkers; additional reviews have been provided by Lam and Gray (2003), Au (2004), Bartell (2006), and Schettino et al. (2012). Livingstone et al. (2000) and Dixon et al. (2002) reviewed biomarkers commonly used to assess pollutant effects in marine invertebrates, including mussels.

Although molecular effects of most chemical compounds on marine organisms and communities are still poorly understood, in recent years there has been a tremendous interest and effort in “omics” technologies to develop biomarkers at the molecular level (Veldhoen et al. 2012, Suárez-Ulloa et al. 2013, Biales et al. 2015). Omics approaches include transcriptomics (identification and quantification of messenger RNA from particular genes), proteomics

(identification and quantification of the complete complement of proteins), and metabolomics (identification and quantification of the small molecule metabolic products) (see section below on “Transcriptomics” for further discussion).

Transcriptomics, the molecular investigation of alterations of expressed genes, offers a new approach for assessing mechanisms by which stressors may affect organisms. Gene transcription is the process by which information from the DNA template of a particular gene is transcribed into messenger RNA (mRNA) and eventually translated into a functional protein. The amount of mRNA transcribed from a particular gene is physiologically dictated by a number of intrinsic and extrinsic factors, including stimuli such as infectious agents, toxin exposure, trauma, or neoplasia. As a result of this keystone function, analysis of mRNA can provide information about dynamic changes in the physiological state of an organism. Alterations (including up-regulation, or increased mRNA, and down-regulation, or decreased mRNA) of transcribed genes indicative of physiological processes at the cellular level can be particularly useful to identify exposure and elucidate mechanisms by which contaminants may have deleterious effects (Snell et al. 2003, McLoughlin et al. 2006, Mos et al. 2008, Wu et al. 2008, Poynton and Vulpe 2009, Miller et al. 2011, Evans and Hofmann 2012).

Herein, we review biomarkers that have proven important in assessing exposure to and genotoxic effects of PAHs in bivalves including mussels, and then provide a detailed review of newer methods to assess exposure and effects, based on evolving genomic technologies.

Mussels as a Sentinel Species

Mussel is the common name for members of a group of taxonomic families comprising bivalve molluscs with elongated shells. They are a ubiquitous component of nearshore marine areas in almost all coastal regions of the world. They are sessile, filter-feeding organisms, traits which make them particularly susceptible to pollution, as they can take up contaminants from sediments, suspended particles, the water column and through their food. Further, mussels tend to bioaccumulate xenobiotic compounds, and thus provide a tool for directly quantifying contaminants. Their abundance and presence in nearshore areas makes them readily available for sampling. Mussels are a critical species in nearshore food webs as a prey resource for many higher trophic levels, and generally are an important resource for human consumption as well.

Mussels have proven to be highly valuable as bioindicator species for monitoring contaminants. Globally, various members of the family Mytilidae are used widely in contaminant studies, including those of the genus *Mytilus* (*M. edulis*, the blue or common mussel; *M. trossulus*, the bay mussel; *M. galloprovincialis*, the Mediterranean mussel; and *M. californianus*, the California mussel), the genus *Perna* (*P. perna*, *P. viridis*, and *P. canaliculis*), and the genus *Modiolis* (*M. modiolis*, the horse mussel). Of note, mussels (*Bathymodiolus azoricus*) that inhabit deep-sea hydrothermal vents have also been the object of considerable research, including a number of gene expression studies, because of interest in their physiological adaptations and symbiotic relationships (Bettencourt et al. 2010).

Because of their utility as a bioindicator, mussels have been the foundation species in an international marine monitoring program, “Mussel Watch.” The program was initiated in the USA in the 1970s, and now includes monitoring at over 300 sites, measuring over 140 analytes, including metals and organic compounds, in tissues of *Mytilus* species or, where mussels are less available, oysters (*Crassostrea virginica*) (Kimbrough et al. 2008; <http://ccma.nos.noaa.gov/about/coast/nsandt/musselwatch.aspx>). The program has expanded globally, and there is now an international network of monitoring sites (Monirith et al. 2003, Rodriguez y Baena and Thébault 2007, Sparks et al. 2014, http://ccma.nos.noaa.gov/stressors/pollution/nsandt/musselwatch/as_intl_mw_study.aspx).

Findings

MFO detoxification systems in invertebrates

Mixed-function oxidases (MFO) are enzymes important to metabolic detoxification in organisms exposed to contaminants. Most studied of these are the cytochrome P450 superfamily of enzymes (CYP), particularly CYP1A1, a biomarker of exposure to aromatic hydrocarbons and other organic xenobiotics in vertebrates (Payne 1987, Stegeman and Lech 1991). Extensive efforts have been made to identify comparable detoxification systems in invertebrates; mussels, in particular, have been commonly used as a bioindicator species. Reviews of cytochrome P450 enzymes in invertebrates (Moore et al. 1980, James 1989, Stegeman and Lech 1991, Livingstone 1998, Snyder 2000, Rebelo et al. 2003, Rewitz et al. 2006) have presented similar general conclusions. Cytochrome P450 proteins are widespread in marine invertebrates, and although many CYP proteins are recognized to have a detoxification role in these taxa, knowledge of specific proteins involved, control of induction, and rates of transformation are lacking. Metabolic processes appear to differ, and rates of transformation of xenobiotics to be slower, in aquatic invertebrates compared to vertebrates. These factors likely contribute to a greater bioaccumulation of xenobiotic compounds in invertebrates, and thus to their value for direct analyses of contaminants.

Numerous studies have sought to characterize specific CYP proteins in mussels and quantify levels of induction following exposure to aromatic hydrocarbons (Wootton et al. 1995, Shaw et al. 2000, 2002, 2004, Chaty et al. 2004, Solé and Livingstone 2005, Cubero-Leon et al. 2012). Most recently, Zanette et al. (2010) used genome sequencing to identify 58 CYP genes in *M. californianus*. Further work by Zanette et al. (2013) examined expression of two CYP1-like and three CYP3-like genes in different tissues of *M. edulis* in response to several known AHR agonists. They found no change in expression of the CYP1-like genes following exposures, but did find evidence of increased CYP3-like gene expression in some tissues following exposure. Significant variation among tissues in expression of CYP genes in an oyster, *Crassostrea brasiliana*, also was reported by Luchman et al. (2014). In general, further study of the full

complement of CYP genes in mussels and other bivalves is needed to understand expression, regulation, and functions of the individual CYPs.

Genetic technologies to monitor genotoxic effects of PAHs

Genotoxicity refers to the potentially harmful effects on genetic material that occur as a consequence of induced damage to DNA following exposure to an agent (e.g., a chemical compound, or radiation). Genotoxic effects may have significant ecological relevance as they are implicated in many pathological processes, and can exert effects beyond that of the individual, potentially carrying through multiple generations (Depledge 1998, Jha 2004, Bolognesi and Cirillo 2014). Genotoxicity effects can occur from endogenous sources (e.g., the attack of reactive oxygen species [ROS] and free radicals, produced as by-products in normal metabolic processes), or exogenous sources (e.g., exposure to radiation, natural toxins, or anthropogenic chemicals) (De Flora et al. 1991). Chemical compounds that damage DNA can act directly on DNA, produce metabolites that cause DNA damage, increase the production of ROS, or inhibit DNA synthesis and repair (Lee and Steinert 2003). These can result in formation of DNA-adducts with toxic molecules, secondary alterations of DNA via ROS production, and alteration of cell functions. Potential consequences include impaired enzyme function, cytotoxicity, immunotoxicity, reproduction disturbances, growth inhibition, and development and progression of diseases such as cancer (Mastrangelo et al. 1996, Ohe et al. 2004, Monserrat et al. 2007). Certain PAHs are of particular concern in genotoxicity due to their potential mutagenic and carcinogenic features (Baršienė et al. 2012).

The two most widely used techniques for assessment of DNA damage are (1) the comet assay and (2) the micronuclei test (Dixon et al. 2002, Bolognesi and Cirillo 2014). The comet assay, also called single-cell gel electrophoresis (SCGE), is a rapid and sensitive technique for detecting DNA strand breaks, and is considered an excellent genotoxicity biomarker to detect a broad spectrum of DNA lesions, with very high sensitivity (Fairbairn et al. 1995, Collins 2004, Jha 2008, Dhawan et al. 2009, Frenzilli et al. 2009, Frenzilli and Lyons 2013). This assay has been widely applied to detect DNA damage associated with PAH exposure in aquatic invertebrates including mussels (see for example Hamoutene et al. 2002, Large et al. 2002, and Thomas et al. 2007; and reviews by Livingstone et al. 2000, Dixon et al. 2002, Bolognesi and Cirillo 2014). Bolognesi and Cirillo (2014) provide a recent summary of studies of DNA damage evaluated by the comet assay in mussels (*Mytilus* spp) (Table 1).

Table 1. Summary of studies assessing DNA damage by the comet assay on mussel (*Mytilus* spp) (adapted and modified from Bolognesi and Cirillo, 2014)

Animal	Target Tissue	Contaminants	Reference
<i>M. edulis</i>	Haemocytes	Alpha, beta and gamma-emitting radionuclides	Alamri et al. 2012
<i>M. edulis</i>	Digestive gland	PAHs	Shaw et al. 2004
<i>M. edulis</i>	Haemocytes	Heavy metals	Dallas et al. 2013
<i>M. edulis</i>	Haemocytes, gill	Wastewater	Rank et al. 2005
<i>M. edulis</i>	Gill	Organophosphates pesticides	Rank et al. 2007
<i>M. edulis</i>	Gill	Heavy metals, PCBs, PAHs, Butylin compounds	Rank 2009
<i>M. edulis</i>	Haemocytes, gill	PAHs	Halldorsson et al. 2004
<i>M. edulis</i>	Haemocytes, sperm	PAHs, heavy metals	Steinert et al. 1998
<i>M. galloprovincialis</i>	Haemocytes	Genotoxic pollution	Klobucar et al. 2008
<i>M. galloprovincialis</i>	Haemocytes	PAHs, heavy metals	Almeida et al. 2013
<i>M. galloprovincialis</i>	Gill	Oil spill	Laffon et al. 2006
<i>M. galloprovincialis</i>	Gill	Industrial and urban wastewater, heavy metals	Nigro et al. 2006
<i>M. galloprovincialis</i>	Gill	PAH, heavy metals	Regoli et al. 2004, Frenzilli et al. 2004

The micronuclei test is a suitable technique to identify and integrate responses to complex mixtures of contaminants, and can act as an index of accumulated genetic damage during the lifespan of the cells (Dixon et al. 2002, Bolognesi and Hayashi 2011). Micronuclei are cytoplasmic masses of unintegrated chromatin that remain in the cytoplasm after cell division (Bolognesi and Cirillo 2014). Micronucleus formation can be the result of a variety of molecular events (e.g., cellular processing of DNA strand breaks, mitotic loss of acentric fragments, or altered segregation of whole chromosomes) (Dolcetti and Venier 2002). Bolognesi and Cirillo (2014) provide a summary of recent studies of micronuclei frequency in mussels (*Mytilus* spp) (Table 2).

Table 2. Summary of studies assessing DNA damage by the micronuclei assay on mussel (*Mytilus* spp) (adapted and modified from Bolognesi and Cirillo, 2014)

Animal	Target Tissue	Contaminants	Reference
<i>M. edulis</i>	Gill	Urban waste	Izquierdo et al. 2003
<i>M. edulis</i>	Gill	Urban waste	Izquierdo et al. 2003
<i>M. edulis</i>	Gill	PAHs, TBT and other organic pollutants	Baršiene et al. 2004
<i>M. edulis</i>	Gill	PCBs, PAHs, chlorinated pesticides	Schiedek et al. 2006
<i>M. edulis</i>	Gill	PCBs, PAHs, chlorinated pesticides	Baršiene et al. 2012
<i>M. edulis</i>	Gill	Oil spill	Baršiene et al. 2006
<i>M. galloprovincialis</i>	Haemocytes, gill	PAHs	Dolcetti and Venier 2002
<i>M. galloprovincialis</i>	Haemocytes, gill	PAHs, PCBs, HCBs	Venier and Zampieron 2005
<i>M. galloprovincialis</i>	Haemocytes, gill	Urban waste	Pampanin et al. 2005
<i>M. galloprovincialis</i>	Gill	PAHs, heavy metals	Bolognesi et al. 2004
<i>M. galloprovincialis</i>	Gill	Oil spill	Bolognesi et al. 2006
<i>M. galloprovincialis</i>	Gill	Industrial activity and urban waste	Nigro et al. 2006
<i>M. galloprovincialis</i>	Haemocytes	Offshore drilling platform	Gorbi et al. 2008
<i>M. galloprovincialis</i>	Haemocytes	Offshore drilling platform	Gomiero et al. 2011
<i>M. galloprovincialis</i>	Haemocytes	PAHs, heavy metals	Bocchetti et al. 2008
<i>M. galloprovincialis</i>	Gill	Heavy metals	Magni et al. 2005
<i>M. galloprovincialis</i>	Haemocytes, gill	Urban and industrial waste (PAHs, PCBs, organochlorinated compounds)	Dalianis et al. 2003
<i>M. galloprovincialis</i>	Gill	Heavy metals	Kalpaxis et al. 2004
<i>M. galloprovincialis</i>	Gill	Heavy metals, PCBs, DDTs, PAHs	Fernandez et al. 2011

The comet assay and micronuclei test have been widely used as biomarkers of genotoxicity in *Mytilus* species. The two genotoxicity assays reflect different biological endpoints, DNA damage (strand breaks) vs. chromosomal damage. The comet assay generally permits detection of a recent exposure, while the micronucleus test can be used to assay for accumulated damage over the lifetime of a cell.

Transcriptomics

Transcriptomics, defined as the study of the transcriptomes and their function (<http://www.nature.com/subjects/transcriptomics>; Brulle et al. 2010, a and Barata 2011), represents one aspect of genomics (i.e., the study of detection of genes), and is a new and rapidly expanding approach to biomarker monitoring. Transcriptomes are the complete set of RNA transcripts that are produced by the genome, under specific circumstances or in a specific cell, and are identified using high-throughput methods such as next generation sequencing (RNAseq). Transcriptomics can effectively provide an early warning system of pathophysiological changes within an organism exposed to biological or physical stressors that include xenobiotics (Poynton and Vulpe 2009, Bourlat et al. 2013). Comparison of transcriptomes provides identification of

genes that are differentially expressed in distinct cell populations, or in response to different treatments or exposures.

For studies of effects of PAHs and other contaminants, transcriptomics involves the analysis of gene transcription changes, measured by the quantity of mRNA from a particular gene, in organisms subjected to differing treatments or environmental conditions. Investigation of differential gene transcription patterns facilitates the discovery of molecular biomarkers of exposure, which in turn can be translated into early indicators of exposure and predictors of physiological effects on individuals, and potentially at the population level as well. Additionally, transcriptomics can help identify the mode of action of stressors by elucidating the particular pathways involved in an organism's cellular and molecular response. Specific gene transcription patterns can be associated with particular stressors or combinations of stressors, and ultimately tied to fitness (Bourlat et al. 2013). A range of technologies have been advanced to investigate gene transcription changes, including RT-PCR, qPCR, microarrays, and RNAseq (see below). The main differences among techniques are the number of transcripts assayed and the intensity of the signal (Bourlat et al. 2013).

Nucleotide sequence data is needed to employ transcriptomic methods. GenBank is an open access database for compilation of sequence data on a wide range of species. Established over 30 years ago, Genbank is maintained by the National Center for Biotechnology Information, U.S. National Library of Medicine (Bethesda, MD, USA (<http://www.ncbi.nlm.nih.gov/>)). Researchers can contribute sequence information determined from their studies, and all sequences are publicly available to other researchers. At present, sequence information is available on over 100,000 distinct organisms (<http://www.ncbi.nlm.nih.gov/genbank>).

Related “omics” technologies that also are advancing include (1) proteomics, defined as the systematic identification and quantification of the complete complement of proteins (the proteome) of a biological system (cell, tissue, organ, biological fluid, or organism) at a specific point in time (http://www.nature.com/subjects/proteomic-analysis?WT.ac=search_subjects_proteomic_analysis) and (2) metabolomics, defined as the systematic identification and quantification of the small molecule metabolic products (the metabolome) of a biological system (cell, tissue, organ, biological fluid, or organism) at a specific point in time (http://www.nature.com/subjects/metabolomics?WT.ac=search_subjects_metabolomics). Although both proteomics and metabolomics are being applied to studies of pollutants and other stressors in mussels (Jonsson et al. 2006, Hines et al. 2007, Hines et al. 2010, Campos et al. 2012, Tomanek 2012, Maria et al. 2013), there currently is much greater research activity utilizing transcriptomics as a tool.

A list of potential candidate genes identified to date that may be of utility in transcriptomics studies to assess response to stress, including exposure to contaminants, is provided in Table 3. These genes were identified from this review of the literature, based on reports on bivalve species.

Table 3. Select genes and functional categories relevant to exposure to a range of stressors, based on gene expression studies in bivalve species.

System	Gene	References
Antioxidant defense	Catalase	Dondero et al. 2006a,b, Veldhoen et al. 2009, Cappello et al. 2013, Giuliani et al. 2013, Lacroix et al. 2014
	Copper/zinc superoxide dismutase	Boutet et al. 2004a, Giuliani et al. 2013, Lacroix et al. 2014
Apoptosis	Caspase 2	Lacroix et al. 2014
	Caspase 8	Lacroix et al. 2014
Cell protection	Heat shock protein 27	Dondero et al. 2006a,b
	Heat shock protein 70	Boutet et al. 2004a, Dondero et al. 2006a,b, Veldhoen et al. 2009, Li et al. 2010
Detoxification	Aryl hydrocarbon receptor	Butler et al. 2001, Liu et al. 2010
	Cytochrome b	Boutet et al. 2004a
	Cytochrome P450	Boutet et al. 2004a, Chaty et al. 2004, Veldhoen et al. 2009, Chen et al. 2012, Miao et al. 2011, Pan et al. 2011, Cappello et al. 2013, Giuliani et al. 2013, Tian et al. 2014, Lacroix et al. 2014
	Glutathione S-transferase	Boutet et al. 2004a, Dondero et al. 2006a,b, Hoarau et al. 2006, Miao et al. 2011, Cappello et al. 2013, Giuliani et al. 2013, Lacroix et al. 2014, Pain-Devin et al. 2014; Lüchmann et al. 2014
	Metallothionein	Dondero et al. 2006a,b, Sureda et al. 2011, Lacroix et al. 2014, Pain-Devin et al. 2014
	Monoamine oxidase A	Boutet et al. 2004b
	P-glycoprotein	Lacroix et al. 2014
Immune function	Defensin	Li et al. 2010
	Lysozyme	Dondero et al. 2006a, Li et al. 2010
	Multicopper oxidase	Bado-Nilles et al. 2010
	Myeloid differentiation primary response 88	Bado-Nilles et al. 2010
	Myticin B	Li et al. 2010
	Myticin A	Dondero et al. 2006a,b
	Mytilin B	Dondero et al. 2006a,b, Li et al. 2010
Reference gene	18S	Dondero et al. 2006a,b, Sureda et al. 2011, Lacroix et al. 2014
	28S	Lacroix et al. 2014
	S4	Veldhoen et al. 2009
	S9	Veldhoen et al. 2009
	Elongation factor 1 α	Bado-Nilles et al. 2010, Lacroix et al. 2014
Tumor suppression	P53	Dondero et al. 2006a,b, Banni et al. 2009, Bado-Nilles et al. 2010, Di et al. 2011, Lacroix et al. 2014

Below, we present results of recent studies on mussels or other bivalves (and in one case, a fish) that have been exposed to hydrocarbon contaminants. Studies are categorized by the

specific approach to measurement of gene expression, including 1) Reverse transcription polymerase chain reaction, 2) Suppression subtractive hybridization, 3) Quantitative polymerase chain reaction, 4) Microarray, 5) Genome elucidation, and 6) RNAseq.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is one of many variations of polymerase chain reaction (PCR). In general, PCR-based techniques amplify stretches of DNA by creating many identical or near-identical copies. RT-PCR is used qualitatively to detect gene transcription through creation of complementary DNA (cDNA) transcripts from RNA; this is not to be confused with real-time quantitative PCR (qPCR; see below) which is used to quantitatively measure the amplification of DNA using fluorescent probes. Although RT-PCR and traditional or standard PCR both produce multiple copies of particular DNA isolates through amplification, the applications of the two techniques are fundamentally different. Traditional PCR is used simply to exponentially amplify given DNA sequences. RT-PCR is used to clone expressed genes by reverse transcribing the RNA of interest into its DNA complement (cDNA) through the use of reverse transcriptase. Subsequently, the newly synthesized cDNA is amplified using traditional PCR and then qualitatively analyzed to determine relative transcript levels.

Mussels and hydrocarbons

Relatively little work has been done using RT-PCR to examine effects of hydrocarbons on mussels primarily because the method is semi-quantitative at best, and other transcriptomic approaches have more merit. Studies that have been done with RT-PCR are limited in scope. Lima et al. (2008) investigated effects of hydrocarbon exposure on cancer processes in *M. galloprovincialis* through examination of ras gene transcription levels. The ras gene is often involved in aberrant cell proliferation, altered cell checkpoint control and cell differentiation, which are carcinogenic manifestations. Mussels were sampled from laboratory experiments in which they were chronically exposed to the water-accommodated fraction (WAF) of fuel oil at varying levels, and from field sites on the northwest coast of Portugal known to be contaminated with petrochemicals. Ras gene transcription levels were measured in digestive gland and gonad tissue. Expression of ras was lower in mussels exposed to PAHs in the field, or to the highest level of WAF in the laboratory study, compared to mussels from a reference site. Overall, expression of the ras gene was higher in digestive glands than gonads. Of note, a mutation in the ras gene was documented in one individual mussel exposed to the WAF.

Glutathione S-transferase (GST) is an enzyme involved in detoxification of many chemical compounds, and as such was selected for investigation into effects of hydrocarbons on *M. galloprovincialis* (Hoarau et al. 2006). Mussels were treated with either cadmium (Cd), the PAH benzo[a]pyrene (BaP) or a combination of the two. The transcription of GST-*pi* gene showed the lowest value in the digestive glands of mussels exposed to BaP, while treatment with cadmium and co-treatment with Cd and BaP elicited higher transcription of GST-*pi* than in

controls. Mussels collected from six sites along the south coast of Portugal also showed different GST- π transcription levels, related to their pollutant content.

M. galloprovincialis was the subject of another study (Cappello et al. 2013) that examined effects of hydrocarbons on cytochrome P450Y1 (CYP4Y1) transcription. Transcription of CYP4Y1 was suppressed in the digestive glands of mussels collected from a pollution-impacted site compared to mussels from a reference site, both on the coastline of Sicily, Italy.

Mussels and other stressors

Franzellitti et al. (2010) assessed effects of multiple stressors on *M. galloprovincialis* in the Adriatic Sea, using both, three established biomarkers (measures of lysosomal membrane stability, lipofuscin content, and metallothionein levels in tissues), and RT-PCR to measure transcription of seven stress-related genes, including metallothioneins (MT10 and MT20; induced by heavy metal exposure), heat shock proteins (HSC70 and HSP70; induced by thermal and other stressors) and Multi Xenobiotic Resistance-related transporters (Pgp, Mrp2, and Mvp; induced by contaminant exposure). Mussels were transplanted to a contaminated site and to a relatively clean reference site, and their digestive glands sampled for gene expression and other biomarkers at 0, 2, 4, 7, 14, and 30 days following transplant. Six of seven targeted genes were significantly up-regulated in mussels from the contaminated site, and one was significantly down-regulated, and the three biomarkers also differed significantly compared to mussels from the reference site. For all seven genes, the changes in gene transcription profiles were detected within two days of exposure, consistent with the assumption that molecular biomarkers are more sensitive and respond much faster to stress of exposure relative to the other three biomarkers, which were not detected until at least 4 days post-exposure. The rapid response of gene transcription profiles to changing environmental conditions and multiple stressors supports their use in field studies as early warning indicators.

Other bivalve species and hydrocarbons

Studies of hydrocarbon exposure in the Pacific oyster, *Crassostrea gigas*, by Boutet et al. (2004a) examined transcription of two non-P450 enzymes, monoamine oxidase A (MAO A) and flavin-containing monooxygenase 2 (FMO-2), both involved in xenobiotic biotransformation. MAO A transcription was up-regulated in digestive glands of oysters exposed to hydrocarbons after 21 days of exposure. FMO-2 was up-regulated in the digestive gland of oysters exposed to hydrocarbons at day-7 compared to control oysters. Boutet et al. (2004b) analyzed transcription of four glutathione-S-transferases (GSTs – omega, mu, pi, sigma) in oysters after hydrocarbon exposure. GSTs are enzymes involved in detoxification of xenobiotics. Results showed up-regulation of omega and mu class GST mRNA transcription in the digestive gland.

Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization (SSH) is a PCR based technique for elucidation of genes that are differentially transcribed between two conditions (Hillmann et al. 2009, Badapanda et al. 2013, Li et al. 2013). This technique is useful for identification of differentially transcribed genes, especially in organisms where genomic data are not available. The SSH method can isolate dozens or hundreds of genes that are differentially transcribed. However this technology is based on hybridization, so that genes that have few or no restriction enzyme cutting sites will not be isolated (Hillmann et al. 2009, Badapanda et al. 2013, Li et al. 2013).

Mussels and hydrocarbons

Adult female *M. edulis* were exposed to styrene (an organic aromatic compound) at 3-5 ug/g for three days with subsequent digestive gland analysis via SSH (Diaz de Cerio et al. 2013). A total of 287 up-regulated clones were sequenced, with a 53% identification rate when compared with Mytibase and DeepSeaVent databases (both mussel-specific gene sequence databases). Widely used stress biomarkers identified were glutathione-S-transferase, heat shock protein 90, and lysosome-related sequences. Additional up-regulation was seen in genes involved in drug response, immune defense, and cell proliferation. Quantitative PCR was performed on selected genes for validation (see qPCR section).

Brown et al. (2006) exposed *M. edulis* to benzo[a]pyrene (BaP) at 1 mg/l for 4 days, and analyzed digestive glands using SSH and a nylon macroarray to measure gene transcription changes. While 112 genes were found to be up-regulated and 25 genes down-regulated, only 87 of these were identified as suitable for use on a macroarray. Transcripts elucidated showed that a wide range of genes responded, including genes involved in general stress, oxidative stress, cell adhesion, transcriptional and translational regulation, transport mechanisms, energy metabolism, cell metabolism, lipid metabolism, protein turnover and activation, and lysosomal activity, indicating that BaP elicits an array of responses. The study demonstrated the value of SSH and a macroarray to identify pollutant-responsive genes in aquatic invertebrates.

Other bivalve species and hydrocarbons

The oyster (*C. brasiliana*) was the subject of study to identify bivalve-specific biomarkers in response to exposure to hydrocarbons (Lüchmann et al. 2012). After 24-hour exposure of oysters to a diesel fuel WAF, digestive glands were removed and SSH was used to construct cDNA libraries of up- and down-regulated genes. Of the 430 genes found to be up-regulated, 259 represented biological processes, 88 represented molecular functions, and 83 were unique to cellular components. Of the 315 genes found to be down-regulated, 203 were involved in biological processes, 63 represented molecular functions, and 49 were unique to cellular components. The most abundant up-regulated transcripts identified were associated with the cytoskeleton, followed by translation and respiratory processes. Validation using qPCR confirmed tests for only one of three up-regulated genes and three of four down-regulated genes,

which represented a potential critical limitation of the SSH technique. In continuing work, Lückmann et al. (2014) exposed oysters to phenanthrene, a major PAH component of crude oil. Ten new genes (6 cytochrome P450 and 4 glutathione S-transferase) involved in xenobiotic biotransformation were found to be upregulated in response to exposure, and were associated with more rapid clearance of phenanthrene. The response was tissue-specific, with gill identified as the tissue most important for xenobiotic metabolism.

Quantitative polymerase chain reaction (qPCR)

Quantitative PCR (qPCR, also called real time qPCR) consists of amplification and quantification of a gene sequence specific to the gene of interest, allowing evaluation of measurable differences between treatments and controls (in contrast to RT-PCR, which is semi-quantitative) (Ginzinger et al. 2002). This method is useful as a monitoring tool as it generates highly sensitive and specific responses to a variety of contaminants. It can assess a multitude of stressors simultaneously and can be used as a screening tool prior to targeted analysis of contaminants or to detect unexpected pressures.

Mussels and hydrocarbons

There have been several investigations of genetic effects of hydrocarbons on mussels using qPCR, although most are confined to one or two genes of interest. Some studies have focused on the effects of hydrocarbons on genes involved in cancer development. For example, Ruiz et al. (2012) sampled blue mussels exposed to hydrocarbons (water soluble fraction of heavy fuel oil and styrene) for five months prior to qPCR analysis of digestive glands for transcription of the ras oncogene, and the growth arrest and DNA damage gene (Gadd45 alpha). Ras was down-regulated in both treatments and Gadd45 alpha was up-regulated in the styrene experiment.

Banni et al. (2009) examined effects of organic compounds on transcription of the p53 gene (more appropriately, p53-like, whereas p53 is the human identified gene associated with tumor suppression) in mussel tissues (gill, mantle, adductor muscle, hemocytes, and digestive gland). The p53 gene is involved in the occurrence of hemocytic neoplasia in mussels. *M. galloprovincialis* and *M. edulis* were exposed to sublethal concentrations of both BaP and North Sea crude oil separately. Exposure to BaP resulted in an initial up-regulation and subsequent (after about 48 hours) down-regulation of p53 in digestive gland tissue, and a large down-regulation in hemocytes, while no modulation was observed in gill, mantle, and muscle. An overall down-regulation of p53 was observed in digestive gland tissues of mussels exposed to North Sea crude oil, a more complex mixture of PAH.

In an investigation of potential mechanisms of malignancy, Di et al. (2011) examined transcription of p53 and the proto-oncogene ras in *M. edulis* exposed to a sublethal concentration (56ug/L) of BaP. Transcription for both genes varied by tissue, consistent with tissue-specific function in response to genotoxic stress. Transcription was significantly upregulated in adductor

muscle and mantle tissue of exposed compared to non-exposed mussels. An increase in DNA strand breaks also was noted for exposed mussels.

Members of the cytochrome P450 family of genes are essential components of cellular detoxification systems and have been the focus of several studies examining transcriptional effects of hydrocarbon exposure. Giuliani et al. (2013), for example, examined the effects of hydrocarbon exposure on transcription of genes for enzymes involved in biotransformation and antioxidant pathways, in *M. galloprovincialis*. Mussels were exposed for four weeks to elutriates of sediments contaminated by a complex mixture of PAHs and trace metals. Mussels exposed to highly polluted sediments exhibited increased transcription of three enzymes (CYP3A1, catalase, and glutathione S-transferase (GST) *pi*) in digestive gland tissue.

Sureda et al. (2011) investigated the transcriptional response of mussels (*M. galloprovincialis*) to the Don Pedro oil spill in Spain. In addition to the oil contamination, the Don Pedro vessel contained batteries from trucks and cars, suggesting potential metal contamination to the surrounding area; metallothioneins were selected as biomarker genes for the dual purpose of potential metal exposure and indicators of oxidative stress. Mussels were sampled one, two, and six months after the Don Pedro accident, at affected and reference sites. Consistent with significantly increased PAH levels in soft tissues one month after the disaster (and returning to normal after six months), transcription of both metallothionein isoforms MT10 and MT 20 were increased in oil-affected mussels and returned to levels of the control mussels one month after the spill.

Quantitative PCR is often used to validate other less-specific, transcriptional evaluation methods. Diaz de Cerio et al. (2013) performed SSH with qPCR validation of differential transcription in *M. edulis* exposed to styrene. Digestive gland tissues of male mussels were analyzed for transcription levels of five target genes. Statistical differences in transcription were observed after three days of styrene exposure: endo-1,3-beta-D-glucanase, mytimycin, and NR1DEF were up-regulated, whereas the unknown MGC001879 transcript was down-regulated. No significant differences were observed for chitinase-1 or heat shock protein 90.

Mussels and other stressors

Quantitative PCR has been used as an investigative tool with mussels and stressors other than hydrocarbons. Of particular current concern are effects of global climate change and ocean acidification on the marine environment. Hüning et al. (2013) examined impacts of seawater acidification on mantle gene transcription patterns of *M. edulis* in the Baltic Sea, using the *M. edulis* mantle tissue transcriptome as a foundation. The impacts of an 8-week acclimation period to four seawater *p*CO₂ treatments on the transcription of several genes (33 genes isolated from the transcriptome) involved in metabolism, calcification, and stress responses were elucidated via qPCR. Transcription of genes involved in energy and protein metabolism were strongly affected by acclimation to moderately elevated CO₂ partial pressures. Transcription of chitinase, implicated in the calcification process, was down-regulated, correlating with a linear decrease in shell growth. Shell matrix protein candidate genes were less affected by *p*CO₂. A compensatory

process to enhance shell protection was indicated by a substantial up-regulation of transcription of tyrosinase, a gene involved in periostracum (outermost layer of the shell) formation.

Mussels also have been used as indicators of potentially harmful wastewater effluent. Veldhoen et al. (2009) examined gene transcription differences in *M. modiolus* exposed to municipal wastewater effluent and those at nearby reference locations. Transcription of catalase and sodium-dependent noradrenaline transporter mRNA were significantly elevated in all three tissues examined (adductor muscle, gonad, hepatopancreas) from mussels located near the wastewater outfall compared with those from the reference site. Upregulated transcription at the wastewater location was also found for a member of the ATP-binding cassette transporter family in adductor muscle, and heat shock protein 70 in hepatopancreas.

Li et al. (2010) tested immune systems of *M. galloprovincialis* collected in three mussel farming areas (i.e., three mussel groups, in Spain, Italy and France) in response to an injection of one of three bacterial species (*Vibrio splendidus* LGP32, *Vibrio anguillarum*, or *Micrococcus lysodeikticus*), and to heat or cold stress. To compare transcription patterns between mussels, qPCR was used with four immune genes of interest (defensin, mytilin B, myticin B, lysozyme) and heat shock protein 70 (hsp70). Gene transcription levels varied according to mussel group as well as to the bacterial challenge. The most frequent effect of bacterial injections was down-regulation, especially for mytilin B and myticin B. Heat stress enhanced transcript levels, but no effect was noted for cold stress. Results demonstrated that expression of immune function genes depended on origin of the mussels assayed, and support the importance of gene-environment interactions.

Other bivalve species and hydrocarbons

Many bivalve species have been used in conjunction with qPCR to examine effects of hydrocarbons in the marine intertidal environment. Liu et al. (2010) and Pan et al. (2011) examined effects of hydrocarbons on genes encoding the arylhydrocarbon receptor (AHR) and cytochrome P450 4 (CYP4), respectively, in clams (*Ruditapes philippinarum*). Liu et al. found that at 0.01 ug/L BaP exposure, AHR transcription was up-regulated at 10 days, while at 0.2 ug/L BaP exposure, the transcription of AHR was significantly up-regulated after only 3 days. Pan et al. (2011) found that exposure at 0.01 ug/L BaP elicited no change in CYP4 transcription in clam tissues at 0, 3, or 10 days, whereas at 0.2 ug/L BaP, significant up-regulation of CYP4 transcription in digestive glands was seen after 10 days of exposure. Gill tissues from the same clams did not show CYP4 up-regulation.

Quantitative PCR was used to assess transcriptional changes in CYP3 and CYP1-like genes in scallops (*Chlamys farreri*) exposed to BaP for 10 days (Tian et al. 2014). Although no induction of CYP3-like genes was observed, CYP1-like genes were induced by 0.025 ug/L BaP. In another study on scallops, Miao et al. (2011) found CYP4 transcription was significantly down-regulated in gill and digestive gland, while GST *pi* transcription was up-regulated in response to BaP exposures.

Although *C. gigas* have been studied extensively, very little information exists about effects of hydrocarbon exposure using qPCR. Bado-Nilles et al. (2010) demonstrated transcript modulation of four immune-related genes (multicopper oxidase, macrophage expressed protein 1-like, MyD88 adaptor, and immunoglobulin domain cell adhesion) and the p53-like tumor suppressor gene, in oysters exposed to light cycle oil. Oysters were exposed to oil for seven days and allowed a two week post-exposure recovery. Immediately after the seven day exposure period, transcription of two genes was significantly up-regulated compared to control oysters. After 7 days of recovery, these genes were equal to or down-regulated compared to controls, and after 14 days (the last day of recovery period), three genes were up-regulated and two genes were down-regulated compared to controls.

Microarray

Most gene expression studies on bivalves have relied on microarray technologies (Suárez-Ulloa et al. 2013). DNA microarrays are coated solid surfaces on which a large number of DNA sequences can be spotted. Each DNA spot is specific for a gene. cDNA from a sample is fluorescently labelled and incubated with the microarray chip. When the labelled sample hybridizes with a DNA spot, the complex fluoresces in UV light, which can then be quantitated. Microarrays have been used primarily in mussels to study large scale transcriptional responses to different environmental stress factors (Manfrin et al. 2010, Dondero et al. 2006a, b, Venier et al. 2006, 2011).

Mussels and hydrocarbons

Dondero et al. (2006a) designed and developed a low-density DNA microarray, which included genes whose sequences were already available in Genbank, as well as newly identified genes, to evaluate whether changes in gene transcription occur in *M. edulis* after exposure to North Sea oil hydrocarbons. They found down-regulation of genes involved in immune defense: antimicrobial peptide precursor mytilin-b, lysozyme, actin, and abc transporter mrp2. Other significantly down-regulated genes were hsp70, major vault protein mbp gene, metallothioneins MT10 and MT20, the RNA helicases, and the p53-like gene. Only catalase showed a significant up-regulation in transcription. These results were validated by qPCR.

Venier et al. (2006) created MytArray 1.0, a cDNA microarray of *M. galloprovincialis*, including 1714 mussel probes. Mixtures of heavy metals or organic contaminants were tested on different groups of mussels, and different tissues were evaluated. Analysis of the transcription changes of specific genes in digestive gland tissue allowed for discrimination of mussels treated with heavy metals or organic contaminants. Similar analyses of gene transcription demonstrated a distinction between mussels living in an area polluted by petrochemicals and those in a cleaner reference area.

Mussels and other stressors

M. galloprovincialis is the target organism in studies focused on the transcriptional responses of mussels exposed to a number of seawater pollutants (Venier et al. 2006; Varotto et al. 2013) using a microarray platform (MytArray V1.0, V1.1). This platform also has been used to study gene transcription during annual cycles, and discriminates between sexes (Banni et al. 2011). Other microarray platforms such as the Mussel Immunochip and HMS/SomeroLab-Mytilus-105K have been used to assess effects of different environmental conditions such as infectious processes (Venier et al. 2011) or physical-chemical stress (Lockwood et al. 2010). Additionally, a *M. galloprovincialis* microarray is being developed to study environmentally relevant biotoxins (Suárez-Ulloa et al. 2013). Microarray technologies also have been used to examine effects of exposure to pesticides, heavy metals, or okadaic acid on *M. galloprovincialis* (Manfrin et al. 2010, Dondero et al. 2011).

More specifically, Dondero et al. (2006b) used a low-density DNA microarray, which includes genes whose sequences were already available in Genbank as well as newly identified genes, to evaluate whether changes in gene transcription occur in *M. galloprovincialis* after exposure to mercury. As expected, there was up-regulation of the two metallothionein genes, MT10 and MT20, both involved in heavy metal homeostasis and detoxification. There also was up-regulation of histone H2A, while histone H1 was down-regulated. Down-regulation of genes involved in the humoral immune response occurred for lysozyme, mytilin-b, myticin-a, and in detoxification in glutathion-S-transferase-*pi*, and ABC transporter MRP2. Actin also was markedly down-regulated by mercury.

Mussels inhabit relatively variable and unpredictable habitats, experiencing marked fluctuations in temperature, salinity, oxygen, and food availability due to diurnal, tidal, and climatic cycles. Connor and Gracey (2011) used microarray technology to analyze rhythms of gene transcription in *M. californianus* at different phases in the tidal cycle. Their results indicated that intertidal mussels exist in several distinct physiological states, including a metabolism and respiration phase, a cell-division phase, and a heat stress-response phase (linked to moderate and severe heat stress events).

Genome elucidation (454 or pyrosequencing)

Pyrosequencing is a method of DNA sequence detection technology that enables rapid and accurate quantification of sequence variation. Applications include whole genome sequencing to obtain transcriptomes. An understanding of the biology of mussel species requires defining basic processes, including toxic responses, reproduction, speciation mechanisms, and adaptation to stressors; these processes will be more readily addressed if the full transcriptomes of mussel species are available (Margulies et al. 2005).

Mussels and other stressors

Pyrosequencing was utilized by Craft et al. (2010) to create a database of transcribed genetic transcripts from *M. galloprovincialis*. Transcripts were isolated from the digestive gland, foot, gill, and mantle of male and female mussels, with a total of 175,547 sequences obtained. Ninety percent of the sequences could be assembled into contiguous fragments for the foot and mantle, compared to 75% for the digestive gland and gill. Transcripts relating to protein metabolism and respiration, including ribosomal proteins, cytochrome oxidases, and NADH dehydrogenase subunits, were most common. Variation among tissues was identified in transcripts associated with mitochondrial energy metabolism, with the digestive gland and gill having the greatest transcript abundance. In addition, taxonomic profiling of the tissues indicated the presence of an abundant microbial flora associated with the digestive gland.

Philipp et al. (2012) generated a transcriptome database from immune-challenged and stress-treated *M. edulis* from the Baltic Sea, using 454 sequencing of different tissues, with the goal of understanding the immune system as well as specific acclimation and adaptation processes to local and changing environmental conditions.

RNAseq

RNAseq allows more precise measurement of transcript levels than other methods, providing unbiased and extensive information on gene transcription (Suárez-Ulloa et al. 2013). RNAseq utilizes recent advances in sequencing technologies that generate large amounts of high-throughput sequencing data at relatively low cost, essentially allowing gene transcription to be quantified by sequencing and counting the number of individual transcripts present for each gene. In contrast to microarrays, RNAseq has no constraints on the number of targets and requires minimal knowledge of the target organism's genome. Thus it is appropriate for developing techniques in species which have had little or no previous study, and in systems where there may be limited availability of sentinel or model species (Bourlat et al. 2013).

Other species and hydrocarbons

The effects of a complex mixture of pollutants from the April 2010 Deepwater Horizon oil spill on the salt march minnow (*Fundulus grandis*) was investigated by Garcia et al. (2012). Using RNAseq, they found 1070 significantly down-regulated genes and 1251 significantly up-regulated genes in exposed minnows. As expected, results indicated a multifaceted and complex response to oil exposure. Alterations in expression were seen for genes traditionally involved in oil detoxification, including arylhydrocarbon (AHR)-mediated response and CYP genes, and for immune function genes, hypoxia genes and genes in the choriogenin family.

Needs in mussel genomics/contaminants research

A central need in biomarker research, including specifically in the area of transcriptomics, is linking expression of specific genes or panels of genes with exposure to specific contaminants, classes of contaminants, and mixtures of contaminants. Further, if these methodologies are to be of value, effects of multiple stressors (including chemical and physical) on gene expression must be evaluated. Additionally, studies must demonstrate an association between the expression of specific genes and toxic effects that influence the performance (e.g., growth, survival, or reproduction) of an exposed individual and consequences of changes at the individual level for the overall population.

In general, there has been a dearth of investigations into the genetic response of mussels exposed to hydrocarbons in combination with other stressors. In a natural setting, organisms must respond to the direct toxic effects of PAHs and other potential xenobiotics that may have harmful effects, and to additional stressors (e.g., nutrition, temperature, predation) not present under optimal laboratory conditions. The combination of stressors may interfere with functions that normally enable physiological compensation for suboptimal conditions (Billiard et al. 2008, Whitehead 2013). These multi-stressor effects may amplify the metabolic costs of PAH exposures to organisms, and contribute to impacts on individual and population fitness not predictable from direct toxicity of specific hydrocarbon compounds alone (Whitehead 2013). Additive or synergistic interactions among common natural stressors, including pathogens, may significantly amplify the risk of chemically-induced damage (Sih et al. 2004). Examples of population level impacts where deleterious effects may have been elevated by synergistic exposures to hydrocarbons and other stressors include the collapse of the herring fishery in Prince William Sound after the EVOS (Rice 2009) and the dolphin strandings in the northern Gulf of Mexico following the Deepwater Horizon oil spill (Carmichael et al. 2012, Whitehead 2013).

A notable gap in research is the current lack of RNAseq projects specifically addressing effects of hydrocarbons or other contaminants on mussels. This is not surprising, as RNAseq is a very new methodology. Studies utilizing this approach will be critically important to increased understanding of subtle and chronic effects of low-level exposure to PAH and other contaminants on mussels and other invertebrate species, as well as to identification of key biomarker genes for monitoring efforts.

Research suggestions

New methodologies and approaches

We present two primary conclusions regarding detection of effects of PAH exposure to mussels: (1) investigations using standard and widely accepted diagnostics (including chemical analyses of tissues and other biomarker assays) may not be as sensitive as newer genomic approaches, and (2) although advanced technologies will provide far better resolution of physiologic and thus potential ecosystem perturbations, the post-event timing of most studies

may prohibit clear insight into the physiological and ecological pathways involved. Even with the major advances in technology achieved over the past decade, the current paradigm of investigations ‘after the fact’ has limitations. Gene transcription technology will allow for implementation of a system of surveillance and investigation that can assess a relatively large number of genes simultaneously, providing information on a broad range of stressors affecting multiple aspects of physiological function, and be incorporated as part of a routine monitoring program. The challenges will be to delineate linkages among environmental stressors (including multiple stressors), expression of specific genes, function of those genes, and consequences for fitness of individuals and populations.

Baseline data (reference ranges)

The advantage of using gene transcript analyses in marine mussel diagnostics lies in the capacity to measure physiologic responses (acute or chronic) of an individual to environmental stressors. Altered levels of gene transcripts are expected to provide the earliest possible indication of health impairment, evident prior to clinical manifestation. However, a key requirement for application of gene transcript technology in monitoring programs will be the establishment of normal or “reference” ranges of values, distinguishing between natural variation in gene transcript levels among healthy individuals and changes associated with exposure and compromised health. Reference ranges for gene transcript analyses in mussel populations are lacking, and need further definition. Baseline data must be collected to ascertain influences of factors including sex, age, tissue, season, and microhabitat effects (see for example Place et al. 2008, Craft et al. 2010, Banni et al. 2011, Izagirre et al. 2014, Lockwood et al. 2015).

Controlled laboratory studies

While effects of multiple stressors found in natural environments cannot be replicated in controlled laboratory studies, necessary baseline information obtained with a select few variables is a critical foundation for future efforts. A two-faceted approach would be of value: (1) single-factor exposure studies targeting different variables: dose and time, pre-and post-exposure, and exposure at different life stages and sexes, and (2) studies with multiple biomarkers and physiological assays to link gene transcription findings to more traditional approaches, and to effects on the individual organism.

Interactive Stressors

An understanding of interactions of multiple stressors on organism health and transcription of various genes will be critical for interpretation of findings. Continued transcription of genes responsible for immunologic function, including detoxification, can be physiologically costly. Reallocation of nutrients and energy from one portion of an individual’s resource budget to other functions can have multiple effects. Mitigation of detrimental effects may impose metabolic and physiological demands on the individual above those normally

required to sustain life, with potential for decreased reproductive capability, increased susceptibility to disease, or disadvantageous behavioral changes.

Numerous studies have been designed using an integrated approach, with a series of biomarkers to examine effects of multiple stressors on mussels and other bivalves (see for example Dondero et al. 2006a, Shaw et al. 2011, Marigómez et al. 2013, Brenner et al. 2014, Ellis et al. 2014, Izagirre et al. 2014). Gene transcription approaches are poised to contribute substantially (and potentially as a replacement for many established biomarkers) in the understanding and interpretation of multiple stressors.

Ultimate goal

The culmination of these efforts would be a comprehensive system of marine monitoring and surveillance based on emerging gene expression technologies, using mussels and possibly other marine invertebrates as bioindicator species. This, in combination with monitoring of population and nearshore community metrics, could provide an early warning system for populations and ecosystems at risk from contaminants and other environmental changes.

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