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Assessing legacy and endocrine disrupting pollutants in Boston Harbor with transcriptomic biomarkers

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ABSTRACT

Within monitoring frameworks, biomarkers provide several benefits because they serve as intermediates between pollutant exposure and effects, and integrate the responses of contaminants that operate through the same mechanism of action. This study was designed to verify the use of transcriptomic biomarkers developed in our prior work (i.e., Coastal Biosensor of Endocrine Disruption; C-BED assay) on *Mytilus edulis* and identify additional biomarkers for legacy pollutants. *M. edulis* were collected from a reference site in Pemaquid, ME, USA and deployed by the Massachusetts Water Resources Authority (MWRA) at locations in and outside Boston Harbor, MA, USA: including (1) Boston Inner Harbor (IH), (2) the current outfall (OS), (3) 1 km away from the current outfall (LNB), and (4) Deer Island (DI), the site where untreated wastewater was formerly discharged into the bay. Differential gene expression was quantified with a high density microarray. Seven genes significantly correlated with whole tissue concentration of PAHs, and six genes significantly correlated with whole body concentrations of PCBs, two groups of legacy contaminants that were elevated at stations IH, OS, and DI. Enrichment analysis indicated that IH mussels had the highest induction of stress response genes, which correlated with the higher levels of contaminants measured at this site. Based on the C-BED assay gene analysis, stations IH and OS exhibited signs of endocrine disruption, which were further confirmed by incorporating the results for the C-BED assay within the Integrated Biomarker Response (IBR) approach. This study successfully demonstrated the potential use of transcriptomic biomarkers within a monitoring program to identify the presence and organismal responses to endocrine disrupting and legacy contaminant classes.

1. Introduction

Biomarkers have several advantages in environmental monitoring as they integrate exposure through all sources and routes (dermal, diet, inhalation), are sensitive, and provide information on the bioavailability of contaminants (van der Oost et al., 2003; Hook et al., 2014). Boston Harbor, MA, USA provides a model system for evaluating molecular methods for toxicant exposures as it has a long history of pollution, and has an existing, long-term monitoring program with the Massachusetts Water Resource Authority (MWRA). Boston Harbor is also an ideal location for integrating monitoring strategies for Endocrine Disrupting Compounds (EDCs), as these compounds have been documented throughout the area, and have the potential to cause population level effects in wildlife at low exposure levels (e.g., ng/L) (Kidd et al., 2007; Griffith, 2013; Griffith et al., 2016). In our previous study, we introduced the Coastal Biosensor for Endocrine Disruption (C-BED) assay, which was designed based on laboratory exposures to estrogenic compounds and successfully detected EDCs in field collected mussels (Blalock et al., 2018). The goal of this paper is to provide the proof-of-concept for measuring molecular biomarkers within an existing monitoring program, by focusing on a case study in Boston Harbor.

Contaminants can enter Boston Harbor from numerous sources including urban and stormwater run-off, combined sewer overflows (CSOs) and sewage discharges, illicit discharges, and inputs from the Charles and Mystic Rivers (Cantwell et al., 2016). Boston Harbor is an urban estuary and prominent shipping port and may also have residual pollution from historical contamination. For years sewage was discharged directly into the harbor causing environmental degradation and dead zones (Taylor, 2002, 2010; Diaz et al., 2008;,). With its growing population and insufficient wastewater treatment system, Boston became one of the most contaminated harbors in the United States (Parthum, 1970). Over the past thirty years, numerous improvements have been made to the sewage treatment plant including the upgrade to secondary treatment and a 13 km outfall tunnel that terminates in a 2 km long diffuser that disperses effluent into

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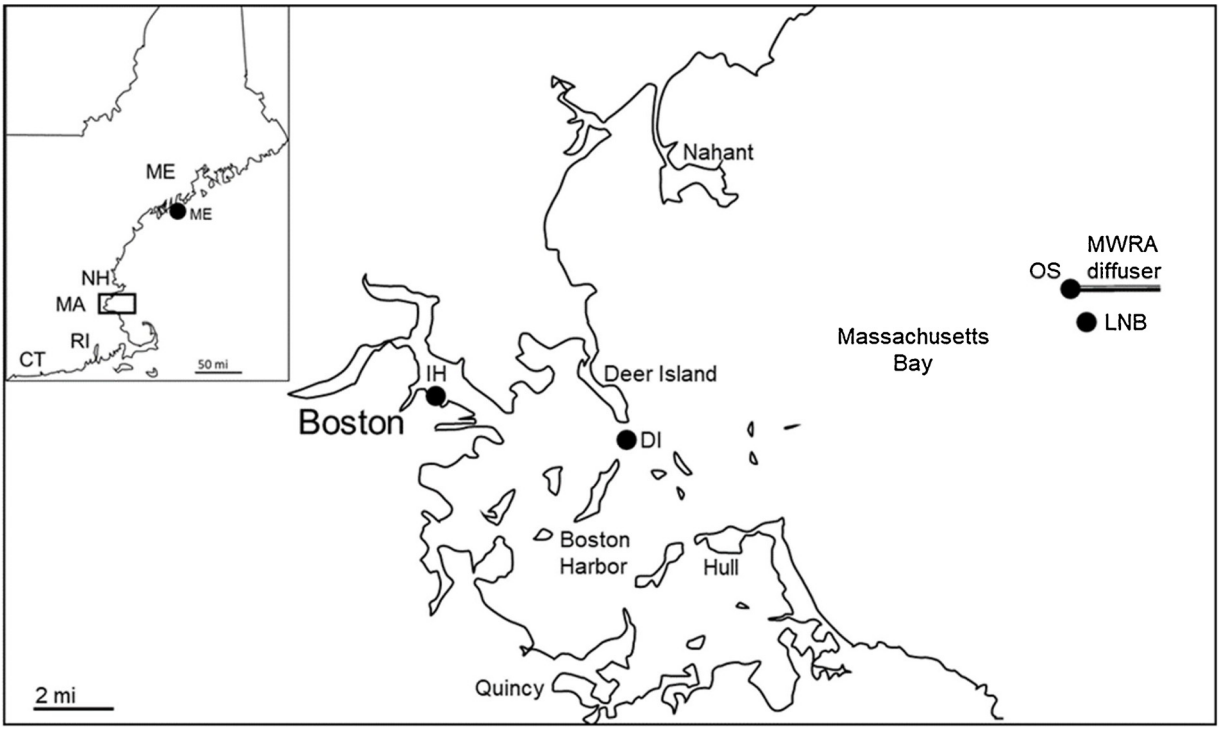

Fig. 1. Inset map of New England, USA showing the location of Pemaquid Point, ME which served as our reference site (ME) and was also the site where deployed mussels were collected. The boxed area around Boston Harbor is enlarged showing the locations of mussels deployed in Massachusetts for 60 days in Boston Inner Harbor (IH), the MWRA outfall (OS), B Buoy (LNB), and Deer Island (DI). The location of the MWRA diffuser pipe is also shown.

Massachusetts Bay (Sherman et al., 1994; Hunt et al., 2010).

To ensure the new treatment plant and outfall are not contributing to the degradation of the harbor, and to monitor the progress of the harbor clean-up efforts, the MWRA was established to evaluate the environmental impact of the outfall on Boston Harbor (Hall et al., 2013; Nestler et al., 2013; Werme et al., 2013). Part of this monitoring program included measuring contaminants in the tissue of key indicator species including mussels (Nestler et al., 2013). Since the wastewater treatment upgrades, there has been a decrease in many contaminants including PAHs, PCBs, DDT, and chlordane, and the condition of the harbor and surrounding beaches have vastly improved (Taylor, 2002, 2010; Hall et al., 2013).

These improvements are consistent with global trends that indicate a general reduction of legacy pollutants (Maruya et al., 2014; Melwani et al., 2014). However, contaminants of emerging concern such as EDCs are increasing in their production and occurrence in marine waters (Dodder et al., 2014; Maruya et al., 2014, 2015). EDCs enter bodies of water primary through wastewater treatment plants and can cause developmental and reproductive impacts to wildlife and humans (Jobling et al., 1998; Benotti et al., 2008; Balabanič et al., 2011). Each day, wastewater from more than 2 million people is pumped to the Deer Island treatment plant and then discharged into Massachusetts Bay. Studies have found that often only 51–67% of estrogenic activity contained in influent wastewater are biodegraded during wastewater treatment processes (Holbrook et al., 2002). Given the prevalence of EDCs and the volume of effluent that is discharged into Massachusetts Bay, it is not surprising that EDCs and natural estrogens have been documented in wastewater effluent and the surrounding bay (Griffith, 2013; Griffith et al., 2016). Effluent concentrations of 17α-ethinylestradiol (EE2) in particular range substantially from 2.7 to 30 ng/L as documented in two studies in 2012 and 2013 (Griffith, 2013; Griffith et al., 2016).

Incorporating molecular tools into biomonitoring, risk assessment, and regulation has been a slow and often controversial process as the results may not be conclusive or are considered difficult to interpret in dynamic field environments (Campos et al., 2018). There is an urgent need to be able to clearly summarize and communicate biomarker results in field environments and rank and categorize ecological risk. The Integrated Biomarker Response (IBR) is one approach that can help simplify the interpretation of multiple biomarkers into a single metric by providing a visual summary with star plots and a numeric value based on the star plot area (Beliaeff and Burgeot, 2002). This approach simplifies the interpretation of multiple biomarker responses which can help improve interpretation and communication to decision makers.

To evaluate the potential risk of EDCs, the C-BED assay biomarkers were incorporated into the IBR approach to determine and rank risk of endocrine disruption at locations throughout Boston Harbor and Massachusetts Bay including (1) Boston Inner Harbor (2) the current outfall (3) 1 km away from the outfall, and (4) Deer Island, the site where untreated wastewater was formerly discharged into the bay. Legacy contaminants were measured in the tissue of bivalves by the MWRA (Nestler et al., 2013) and were correlated with differential gene expression data to identify potential new biomarkers. The impacts of pollution and overall health of the mussels were investigated with global transcriptomics using a high-density microarray and an enrichment analysis.

Overall, this study highlights how different gene expression data analysis approaches can be used to assess the impacts of pollution in marine environments by focusing on three main questions: (1) Are EDCs released from the outfall potentially impacting wildlife in Boston Harbor?; (2) Are there biomarker expression signatures that correlate with contaminant data (both legacy and EDC)?; (3) Do transcriptomic responses suggest mussels may be stressed by contaminants in Boston Harbor?

2. Materials and methods

2.1. Mussel collection and deployment

On 29 June 2012, approximately 1600 mussels (*Mytilus edulis*)

Table 1
Latitude and longitude of mussel collected from Pemaquid Point, ME and deployment locations throughout Massachusetts including Deer Island (DI), Outfall Site (OS), "B" Buoy (LNB), and Boston Inner Harbor (IH). Data from Hall et al. (2013).

Station	Sampling Site	N Latitude	W Longitude	Water Depth	Cage Height Above Bottom
DI	Deer Island Light	42°20.4'	70°57.2'	2-5 m	< 1- 1.5 m
OS	Outfall Site	42°23.2'	70°47.5'	33m	12m
LNB	LNB "B" Buoy	42°22.7'	70°47.1'	33m	12m
IH	Boston Inner Harbor	42°21.5'	71°02.9'	8-11m	1.5-4.5 m
ME	Pemaquid Point, ME	43°52.8'	69°31.1'	native	native

between 55 and 65 mm (2–3 years old) were collected from Pemaquid Point, ME, USA at low tide. Mussels were randomly distributed into cages (approximately 30 animals per cage), and deployed by the MWRA on 1 July 2012 at four sites in Massachusetts Bay; Deer Island (DI), Boston Inner Harbor (IH), Outfall site (OS) and "B" buoy (LNB) (Fig. 1, Table 1; Hall et al., 2013). At the OS, four cages were deployed at various locations approximately 60 m south of the diffuser heads and one approximately 1 km away at LNB (Hall et al., 2013; Nestler et al., 2013).

Mussels were retrieved after 60 days on 30 August 2012 and were examined for accumulation of contaminants (described below) for all locations. Twelve mussels were collected from each site, dissected upon retrieval, and stored in RNA later for transcriptomic analysis. Digestive gland tissue was collected for microarray analysis and mantle tissue was collected for sex identification analysis. Twelve mussels were collected again from Pemaquid Point, ME on 1 September 2012 for comparison with field deployed mussels and were used as the reference location for this study (ME). Mussel lengths were recorded for IH (mean: 63.4 ± 3.6 mm), DI (mean: 61.2 ± 4.1 mm), LNB (mean: 62.6 ± 3.4 mm), and ME (mean: 61.1 ± 4.4 mm).

2.2. Inorganic and organic tissue analysis

Chemical analysis was performed by MWRA Central Laboratory and details can be found in Hall et al. (2013). To summarize, mussels were pooled together in a single conglomerate for inorganic and organic analyses. A total of four replicate samples of 25 individuals were analyzed at DI, IH, and LNB and eight replicates of 25 individuals were evaluated at OS (Hall et al., 2013). Four pooled replicates of ME pre-deployment mussels were also analyzed. Contaminants measured included, PCBs, PAHs, chlordane, DDT, cadmium, mercury, and lead (Hall et al., 2013).

2.3. Lab exposure with 17α-ethinylestradiol (a positive control)

Additional mussels were collected from the ME reference location on the same date as the transplant mussels were collected (i.e., 29 June 2012) to act as a positive control for our Coastal Biosensor for Endocrine Disruption (C-BED) assay. These mussels were transported to the lab and allowed to acclimate for 10 days in seawater at 30 psu and 12° C. After mussels were acclimated for 10 days, 20 mussels (62.6 ± 4.4 mm) were randomly distributed into two 10-gallon tanks with 15 L of filtered seawater. One tank was exposed to 5 ng/L EE2 in ethanol carrier (0.0025% ethanol; a positive control for our C-BED assay); the other was only spiked with the ethanol carrier. Water was changed three times a week and treatment tanks were re-dosed with EE2 and the ethanol carrier. Mussels were fed 10 mL of *Isochrysis galbana* and *Thalassosira weissflogii* 2 to 6 h prior to each exposure water change. After 60 days of exposure, mussels were collected, mantle and digestive gland tissue were dissected, and tissues were stored in RNA later.

2.4. RNA isolation

Total RNA was isolated from the mantle tissue (n = 12 per site) for sex identification and the digestive gland of male mussels (n = 3 per site) for microarray analysis. Sex identification was performed at IH, DI, OS, and LNB while microarray analysis was performed at IH, DI, and OS. Total RNA was isolated with TriReagent (Molecular Research Center, Cincinnati, OH) and homogenized for 10 min at 50 vibrations/s using a Tissue Lyser bead mill (Qiagen Inc., Valencia, CA) with 3.2 mm stainless steel beads (Biospec Products, Bartlesville, OK). Total RNA was treated with DNAse I, and purified using a Qiagen RNeasy kit. RNA concentrations were examined using a NanoDrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, DE) and RNA quality was checked using a 1.5% agarose gel and NorthernMax-Gly buffer (Applied Biosystems/Ambion, Austin, TX). RNA was stored at -80 °C.

2.5. Sex identification of mussels

After total RNA of mantle tissue was isolated, it was then reverse transcribed into cDNA following manufactures protocols using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), oligo dT(23) (New England Biolabs, Ipswich, MA), and random primers (New England Biolabs). After the reverse transcription, an RNA hydrolysis reaction was performed by incubating samples for 15 min at 60 °C with a NAOH/EDTA mixture and neutralizing with Tris buffer (pH 7.4). cDNA was then purified using Amicon Ultra Centrifugal Filters according to manufacturer's protocols (Millipore). Concentration and quality control ratios (260/230, 260/280) were checked with a NanoDrop Spectrophotometer 2000 (Thermo Scientific, Wilmington, DE). cDNA templates were diluted to 5 ng/μL and samples were stored at -20 °C until evaluated by reverse transcription quantitative PCR (RT-qPCR) using the sex identification assay developed by Hines et al. (2007) with slight modifications (Blalock et al., 2018).

2.6. Microarray construction and hybridization

The oligonucleotide microarray was constructed with a previous *de novo* transcriptome assembly using 300 different mussel samples representing different developmental stages, tissues, chemical and stress treatments, and geographic locations (Blalock et al., 2018). The resulting transcriptome contained 39,955 contigs and the microarray was printed using 8 × 60,000 sequence format (AMADID no.066389). Two probes were used for each gene that was functionally annotated with Blast2GO while one probe was used for genes with unknown functions. Details related to the design of the microarray and probe sequences are available at Gene Expression Omnibase (GEO; http://www.ncbi.nlm.nih.gov/geo/) with accession number: GSE111836.

RNA was prepared for microarray analysis using Quick Amp One Color Labeling kit and One-Color RNA Spike-in kit (Agilent Technologies). Labelled cRNA was hybridized to the custom microarray and scanned at five-micron resolution using an Agilent DNA Microarray Scanner G2565CA at the University of Massachusetts Medical School in Worchester, MA. Feature extraction was performed using Feature Extraction Software 11.5 (Agilent Technologies) and intensity values were normalized by quantile normalization with median polish in Arraystar (DNAstar, Madison, Wisconsin). Each treatment was compared to the ME reference site and differentially expressed genes (DEGs) were selected with a 99% confidence interval and > 2-fold change in

Arraystar (DNAstar). We describe our results as differential "gene" expression to differentiate our study from transcriptomic studies that measure alternately spliced transcripts (e.g., Soneson et al., 2015). The probes on our microarray were designed to conserved exons found across the transcripts for each gene; therefore, the probes represent the combined expression of all alternatively spliced transcripts for each gene.

2.7. Microarray analysis

Differentially expressed genes (DEGs) were separated by up or down-regulation in each field comparison and enrichment analysis was performed using Blast2GO (BioBam, Spain). Comparisons were made ($p \leq 0.05$) with the entire set of annotated genes in the microarray and each DEG list to identify overrepresented gene categories.

2.8. Evaluation of endocrine disrupting compounds

Integrated Biomarker Response (IBR) is an approach developed by Beliaeff and Burgeot (2002) which summarizes multiple biomarkers into a visual summary with star plots and a numeric value based on the star plot area. This approach simplifies the interpretation of multiple biomarker responses into a single score which provides advantages for risk assessments, monitoring, or communicating results to environmental managers. To evaluate the potential effects or exposure of EDCs at each of the locations, we used each of the genes from the Coastal Biosensor for Endocrine Disruption (C-BED) assay (Blalock et al., 2018) and determined the IBR at each location to rank endocrine disruption at each location.

The C-BED biomarkers were selected from a previous EDC exposure that identified genes suspected to be related to the toxicological mechanism of action. These genes include (a) genes within the steroidogenesis pathway: Adrenodoxin (Adx), steroidogenic acute regulatory protein (StAR), membrane bound transcription factor peptidase, site 2 (MBTPS2); (b) genes in the non-genomic estrogen signaling pathway and other signaling pathways: caveolin-1 (Cav-1), Guanylate cyclase 32E (GLC32E), Breast carcinoma amplified sequence 3 (BCAS3), and serotonin receptor (SR); (c) and a gene in the cell cycle: cell cycle regulator (Mat89bb) (Blalock et al., 2018). These eight biomarkers were validated with RT-qPCR and were significantly expressed over time (Blalock et al., 2018).

The IBR calculation is based on four steps summarized below and is described in Beliaeff and Burgeot (2002).

1 The data are standardized (X standardized to obtain Y) using $Y = (X\text{-}m)/s$. Where X is the mean biomarker value for a site, m is mean value for all sites for a particular biomarker (general mean) and s is the standard deviation of a biomarker for all sites.
2 For each biomarker Z is then computed where $Z = Y$ or $Z = -Y$. Positive or negative values indicate whether the expressed biological effect is activation or inhibition.
3 The S value (Score) is then computed with $S = Z + |Min|$, where Min is the minimal value observed for all sites for each biomarker.
4 The final step, calculation of the IBR, is summarized by Beliaeff and Burgeot (2002). However, step 4 of the IBR method may be potentially biased as the area and score (S) is based on the organization of the biomarkers and which biomarkers are placed adjacent to each other on the star plots. To overcome this limitation, Devin et al. (2014) suggested a new method that calculates all possible IBR values using permutations. Dr. Simon Devin generously provided an R-based computer code to calculate the median IBR, and determine the order of biomarkers to obtain the median. Compared to the previous method this approach would allow sites to be ranked more accurately while eliminating bias. Finally, the S values were calculated as described in steps 1–3 above (Beliaeff and Burgeot, 2002; Devin et al., 2014) using the microarray gene expression data. The S values were then plotted in the correct order to obtain the median IBR permutation on the star diagram.

2.9. Genes correlated with contaminants

To identify genes that correlated with contaminant data collected by the MWRA, Pearson correlation coefficients (r) and their p-values were generated for each gene x contaminant combination across the four sample sites using Microsoft Excel 2013. For each contaminant, genes were then ranked based on the corresponding p-values and a false discovery rate (FDR) was generated by multiplying the p-value by the gene rank. Genes with an FDR $< 5\%$ were selected as correlated genes.

3. Results

3.1. Overview of gene expression analysis

We measured global gene expression using microarrays to determine if mussels elicited a specific molecular response to chronic pollution exposure in Boston Harbor, MA, USA. We used a two-pronged approach, first measuring targeted expression of C-BED genes, and second correlating global expression with specific contaminant levels measured in the tissues of the mussels. There was a total of 1664 genes differentially expressed, with 1100 of these genes being annotated, when IH, DI, and OS mussels were compared to the ME reference location. 58 additional genes (39 annotated) were differentially expressed in multiple comparisons of IH, DI, and OS with ME. Of the three sites, DI had the most DEGs with 681 up-regulated genes (408 annotated) and 744 down-regulated genes (542 annotated) when compared to ME. For IH there were 76 genes up-regulated (49 annotated) and 77 genes down-regulated (57 annotated) and there were 86 genes up-regulated (58 annotated) and 58 genes down-regulated (35 annotated) at OS.

3.2. Are mussels responding to EDC?

3.2.1. Sex ID analysis

Sex identification was performed on deployed mussels to evaluate sex ratios and whether potential feminization was occurring at sites throughout Boston Harbor. Overall, the RT-qPCR sex identification analysis found the highest percentage of female mussels at the OS (67%) and ME (67%), followed by LNB and DI (50%), and IH (33%) (Fig. 2). However, some locations had mussels whose sex could not be identified using the RT-qPCR assay: ME (8%), DI (33%), and IH (36%). This limited our ability to detect significant alterations in sex ratios. For example, although the OS site had the highest percentage of females, the lowest percentage males was found at DI and ME (17%).

3.2.2. Integrated biomarker response

Of the many genes that were differentially expressed in our microarray analysis, we were particularly interested in the expression of genes which were correlated with EDC exposure in our previous study, the biomarker genes of the C-BED assay (Blalock et al., 2018). The C-BED assay positive control used in the present study, agreed with our earlier work (Blalock et al., 2018). The eight biomarkers developed from the C-BED assay were integrated into star plots and IBR values were calculated to evaluate the potential risk of endocrine disruption at each site. The ME reference location had a median IBR value of 2.0 while the 5 ng/L EE2 treatment had a median IBR value of 8.8 (Fig. 3). The OS had a median IBR of 11.3 which was the highest out of the field deployed locations as well as higher than the 5 ng/L EE2 positive control exposure. IH fell between these values with a median IBR of 6.4. DI was slightly below the ME reference location with a median IBR of 0.4. The value of using the IBR approach is its ability to rank biomarker responses across sites; therefore, by using the C-BED biomarkers with an IBR, we ranked potential risk of endocrine disruption as DI < ME < IH < EE2 positive control < OS (Fig. 3).

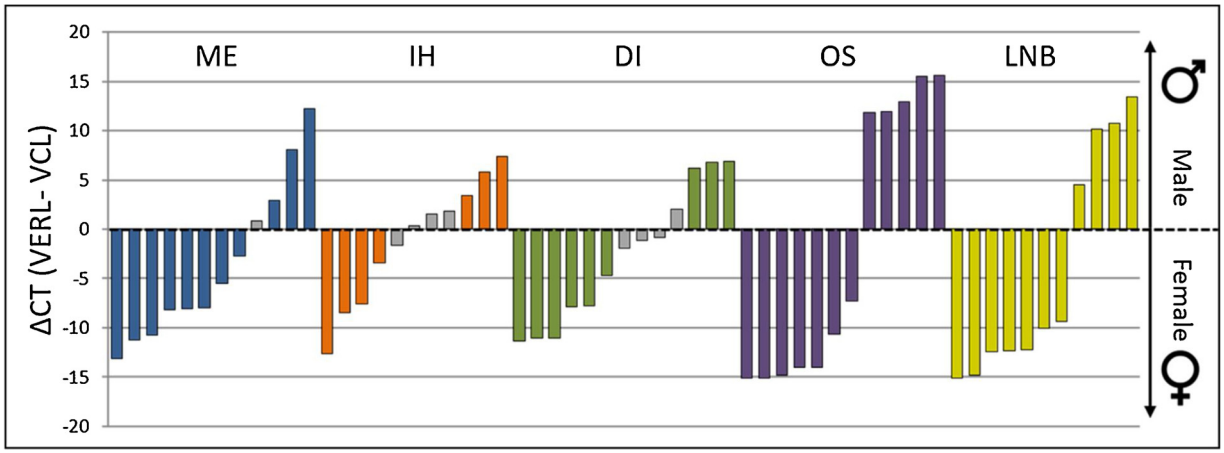

Fig. 2. Results of RT-qPCR sex identification analysis of mussels collected from the reference site in Pemaquid Point, ME (ME) and deployed in Massachusetts for 60 days at Boston Inner Harbor (IH), Deer Island (DI), the MWRA outfall (OS), and B Buoy (LNB). Each line represents one individual mussel with positive values representing male mussels, negative values representing female mussels, and gray lines representing mussels whose sex could not be identified.

3.3. Are there biomarker expression signatures that correlate with contaminant data?

3.3.1. Contaminant concentrations

Several legacy contaminants and metals were measured in the whole body tissues of mussels deployed in Boston Harbor. Overall, ME, LNB, and OS had the lowest concentrations of legacy contaminants followed by DI and IH (Table 2). Several contaminants had similar levels of bioaccumulation across sites including metals (cadmium, lead, and mercury), dichlorodiphenyltrichloroethane (DDT), and chlordane (Hall et al., 2013). Additionally, these contaminant levels are low or close to baseline levels when compared with studies throughout North, Central and South America (Sericano et al., 1995; Sparks et al., 2014).

In contrast, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) had greater variation across locations and were elevated when compared to most locations reported by the Mussel Watch program (Sericano et al., 1995; Sparks et al., 2014). For example, for high molecular weight PAHs (HPAH), concentrations were 14 times higher at IH, 1.3 times higher at the OS, and 1.8 times higher at DI compared to the ME reference location (Hall et al., 2013). Low

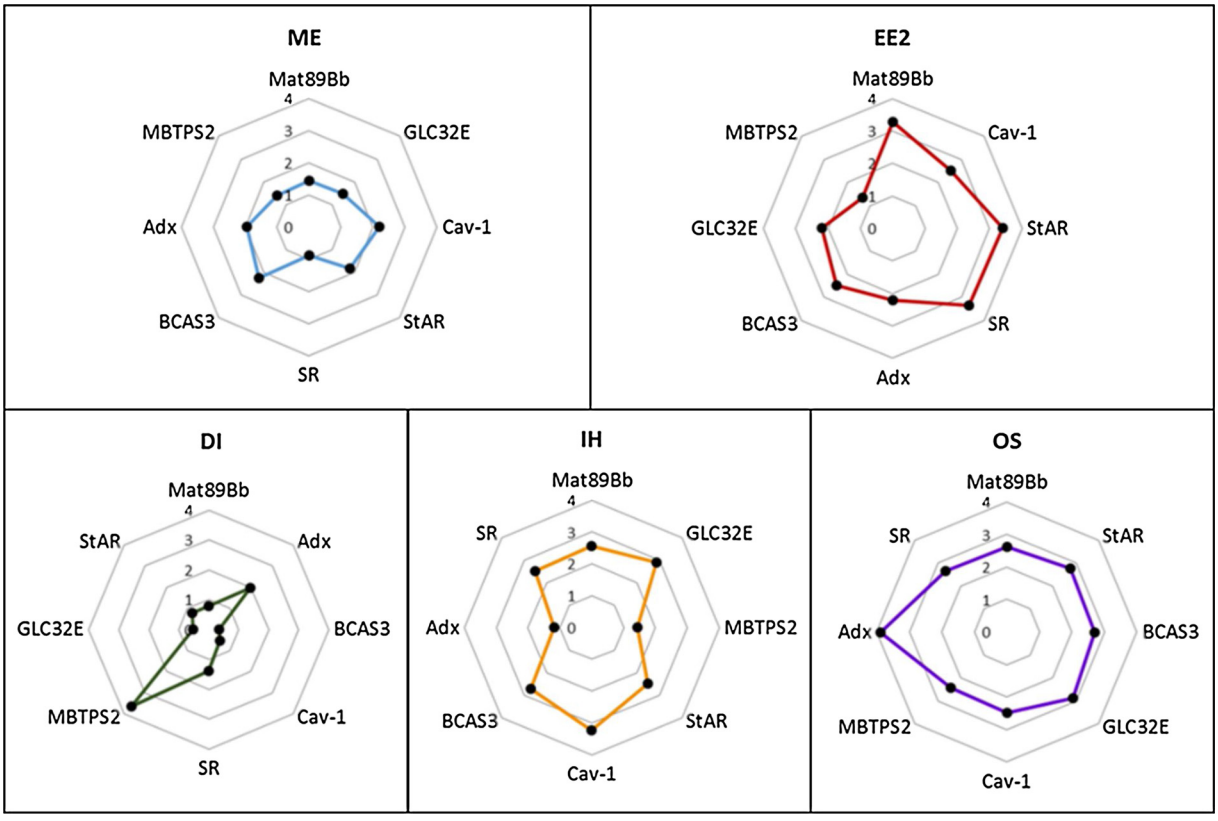

Fig. 3. The IBR of C-BED assay biomarkers from mussels collected from the reference location (ME) and deployed for 60 days at locations in Boston Harbor and Massachusetts Bay (IH, DI, OS). A 60 day lab exposure to 5 ng/L EE2 is also displayed (EE2; positive control). Each axis of the star plot represents the S value of a biomarker from 3 male mussels and are arranged in the correct order to obtain unbiased values for the median IBR permutation. The IBR for ME (2.0), EE2 (8.8), DI (0.4), IH (6.5), and the OS (11.3) was calculated as the total area displayed by the star plot.

Table 2
Concentrations of contaminants accumulated in mussel tissue at Boston Inner Harbor (IH), the MWRA outfall (OS), 1 km away from the outfall (LNB), and Deer Island (DI) after a 60-day deployment and collected from the Pemaquid Point, ME reference population. Data provided by the Maurice Hall of the MWRA and summarized in Hall et al. (2013). ND = not detected.

Contaminant	ME	IH	DI	OS	LNB
Cadmium (μg/gdw)	1.45	1.83	1.43	1.86	1.99
Lead (μg/gdw)	2.50	3.32	2.11	1.84	1.52
Mercury (μg/gdw)	0.13	0.16	0.13	0.14	0.10
Total PCBs (ng/gdw)	4.68	238.28	106.40	20.16	23.78
Total DDT (ng/gdw)	3.18	23.31	8.20	3.14	3.57
Total Chlordanes (ng/gdw)	ND	7.83	4.08	3.88	3.76
HMW PAH (ng/gdw)	48.08	683.43	86.35	60.53	34.99
LMW PAH (ng/gdw)	38.39	108.79	38.96	44.07	28.75

molecular weight PAH (LPAH) concentrations followed a similar pattern where IH was 3 times higher than ME. Similarly, PCBs were 51 times higher at IH, 22.7 time higher at DI, and 4.3 times higher at OS compared to the ME reference location (Hall et al., 2013).

3.3.2. Genes correlated with contaminants

Our approach for identifying candidate biomarkers was to search for genes whose expression pattern significantly correlated with bioaccumulation. Based on MWRA's mussel bioaccumulation data, PAHs and PCBs were most likely to have an influence on transcriptomic responses across sites and we therefore investigated specific genes that correlated with these contaminants.

Using a FDR of 5%, seven genes correlated with both LPAHs and HPAHs (Table 3). All the genes were up-regulated in the correlation analysis suggesting PAH exposure induces expression of these potential biomarkers. The expression of eight genes correlated with LPAH levels including five genes successfully annotated with functions. Finally, six genes were found to correlate with PCBs. As IH was the site with the highest PAH and PCB bioaccumulation, this site likely drove some of the gene expression correlation results. It is possible that other environmental variables or contaminants may be higher at IH and could be responsible for the gene expression correlations.

3.4. Did transcriptomic responses in deployed mussels suggest adverse effects?

Enrichment analysis is a method to assign meaning to a group of genes and identify larger patterns and trends within the data. Enrichment results are represented by GO terms which describes the function of genes through biological processes, molecular functions and cellular components. As we were investigating adverse effects, over-represented biological GO terms were identified at each deployment location to help understand if mussels are experiencing signs of stress (Tables S1-S4). DI had the most enriched GO terms followed by IH and the OS. The enrichment results identified 68 biological GO terms up-regulated and 299 GO terms down-regulated at DI compared to the ME reference site. At IH there were 17 enriched up-regulated GO terms and 43 enriched down-regulated GO terms while the OS only had 1 enriched GO term up-regulated, and 5 GO terms down-regulated.

The enrichment analysis of the DEGs at DI revealed that response to external stimulus and response to nutrient levels were significantly up-regulated GO-terms (Table S1). Additionally, growth was enriched in the up-regulated gene list which agreed with additional data in the MWRA report, finding fish collected off DI were longer and heavier than those from other stations. There was also some suggestion of stress response as double strand break repair was an enriched GO-term in the up-regulated genes at DI. Semaphorin-plexin signaling pathway and the related integrin mediate signaling pathway were both enriched up-regulated GO terms. Semaphorins are extracellular signaling proteins which regulate integrins (Kruger et al., 2005), and transmembrane receptors involved in cell growth, cell division, and apoptosis (Streuli, 2009). Further supporting this trend, cell cycle, and developmental GO-terms were enriched in the data set (Table S1 and S2).

Up-regulated enriched GO terms at IH included intracellular signal transduction and cell communication, cilium assembly, and response to drugs (Table S3). Down-regulated GO terms at IH included aromatic carbon biosynthesis, oxidation reduction, regulation of signal transduction, and negative regulation of cell death. The least number of enriched GO terms were identified at the OS (Table S4). At the OS cell division was an up-regulated GO term while response to endogenous stimulus was down-regulated.

Table 3
Genes correlated (FDR < 5%) with both high and low molecular weight PAHs, high molecular weight PAHs only, low molecular PAHs only, and PCBs after a 60 day deployment with *M. edulis*. Site averages (avg) represent gene expression from 3 replicate male mussels analyzed with microarrays.

Contaminant	Description	FDR %	DI avg	OS avg	ME avg	IH avg
HPAH/LPAH	Unknown function	0.04/4.35	2.32	2.29	2.27	4.13
	Kyphoscoliosis peptidase	1.09/0.76	2.46	2.51	2.34	4.78
	NAD-dependent deacylase sirtuin-	0.66/1.85	2.54	2.55	2.53	3.95
	Rab 6	0.1/3.19	2.32	2.31	2.25	3.26
	Unknown function	2.49/0.07	2.77	2.88	2.63	5.55
	Unknown function	0.81/0.56	2.5	2.57	2.39	5.55
	Unknown function	2.15/0.11	2.25	2.32	2.19	3.94
HPAH	Unknown function	3.36	4.14	4.33	4.45	2.33
	Sulfide:quinone mitochondrial	1.69	2.38	2.32	2.25	3.27
	Unknown function	0.27	2.49	2.38	2.31	4.02
	Unknown function	0.48	2.42	2.24	2.25	4.03
LPAH	calmodulin 3b (phosphorylase delta)	3.59	2.27	2.38	2.36	3.34
	cytoplasmic dynein 2 heavy chain 1-like	0.07	2.53	2.61	2.55	3.59
	macrophage mannose receptor 1-like	1.57	5.79	5.35	5.79	2.6
	hypothetical protein CGI_10023394	0.02	2.8	3.16	2.7	7.18
	tubulin–tyrosine ligase 12	4.96	2.43	2.6	2.32	4.03
	Tyrosine- phosphatase non-receptor type 6	2.73	2.28	2.48	2.32	3.6
	Unknown function	0.26	2.26	2.41	2.32	4.26
	Unknown function	2.46	3.58	3.83	3.68	5.28
PCB	serine threonine- kinase SIK3	0.6	2.8	2.4	2.4	3.5
	integrin-alpha FG-GAP repeat	0.06	7.1	7.8	8	5.7
	cyclin-related FAM58A-like	0.44	2.8	2.4	2.4	3.5
	pol-like protein	0.02	6.1	6.9	7.1	4.8
	Unknown function	1.31	4.4	4.8	5	3.7
	Unknown function	2.46	4.5	3.3	2.8	6.1

4. Discussion

4.1. Are mussels responding to EDCs?

The C-BED assay biomarkers were developed to serve as an early warning indicator for reproductive and developmental impairments. Based on these biomarkers, potential endocrine disruption may occur at the OS as it had a higher IBR score than the 5 ng/L EE2 positive control (11.3 vs. 8.8) and reference site (2.0). The C-BED biomarkers indicated that IH may also be responding to EDCs (median IBR: 6.5) as it had a higher score than the reference site (median IBR: 2.0) and was only slightly less than the 5 ng/L EE2 positive control (median IBR: 8.8). In contrast, DI mussels showed little evidence of endocrine disruption (median: IBR 0.4).

The C-BED assay biomarker response correlates well with expected estrogenic EDC exposures within Massachusetts Bay. In May 2013, the same year as the current study, concentrations of free (E1, E2, E3, EE2), conjugated (sulfate, glucuronide) and halogenated (brominated, chlorinated) estrogens were measured in Deer Island wastewater and Boston Harbor field locations (Griffith, 2013; Griffith et al., 2016). In undiluted Deer Island wastewater effluent, the normalized estrogen concentration represented 14–22 ng/L E2 equivalents while samples within Massachusetts Bay ranged between 0.1 – 0.4 ng/L E2 equivalents (Griffith, 2013; Griffith et al., 2016). These concentrations may vary based on characteristics of Boston wastewater during summer stratified conditions (Hunt et al., 2010).

At the OS, wastewater is released from risers located on the seafloor, just 12 m below where OS mussels were deployed placing mussels at OS in a direct exposure path of the wastewater effluent. The predicted no effect concentration (PNEC) for total steroid estrogens has been estimated to be near 1 ng/L E2 equivalent (Young et al., 2002), and EE2 has an estimated PNEC of 0.1 ng/L (Caldwell et al., 2012). Two synthetic estrogens, 4-(t-octyl)phenol and bisphenol A, were also detected in the sediments and in clams deployed near the outfall diffuser (Stuart et al., 2005). Therefore, the sum of estrogenic compounds may pose a risk to wildlife at the OS, and EE2 specifically is likely contributing greatly to these effects. OS was also the only site where the IBR score was greater than the 5 ng/L EE2 positive control, indicating that not only were mussels at the OS site exposed to EDCs, but they were responding by up-regulating genes within the C-BED assay.

Mussels deployed at DI showed a much lower C-BED response consistent with a high dilution of wastewater effluent. Summer studies in Massachusetts Bay have found that tides and wind have the largest impacts on wastewater dispersion (Tucker et al., 1999; Gustafsson et al., 2001; Hunt et al., 2010) and based on the design and location of the outfall, most concentrations of EDCs should be heavily diluted. Estrogens are pseudo-persistent as they are continually released into the environment although they have a relatively short half-life. EDCs can further degrade in summer months due to photodegredation (Ying et al., 2002; Hernando et al., 2006; Sornalingam et al., 2016). Therefore, estrogen concentrations were likely not at sufficient concentrations to produce strong gene expression changes at DI.

To our knowledge, there are no studies that have measured EDC concentrations or wastewater dilution and dispersion characteristics in inshore coastal waters, therefore, it is not possible to estimate potential EDC exposure to mussels at IH. However, the biomarker response and overall transcriptomic pattern at IH were likely due to other sources than the outfall. The most likely source of EDC exposure is from CSOs (Eganhouse and Sherblom, 2001; Cantwell et al., 2016). By design, CSOs collect rainwater, sewage, and industrial runoff and then transport these waters to a sewage treatment plant for treatment. However, if the volume of wastewater exceeds the capacity (e.g., heavy rainfall events or snowmelt) it discharges directly into neighboring bodies of water. At the time of this study there were 47 active CSOs surrounding the IH deployment location (Coughlin, 2006). Other sources may include illicit discharge of sewage into storm drains, or inputs from the Charles and Mystic River (Cantwell et al., 2016).

4.2. Are there biomarker expression signatures that correlate with contaminant data?

Blue mussels are specialized to quickly filter food particles from the water column and in the process readily bioaccumulate contaminants, making them an excellent species to monitor water pollution (Kimbrough et al., 2008). We used global transcriptomic analysis to determine if particular gene responses correlated with the concentrations of contaminants in mussel tissues. This approach has been employed in several previous studies using multiple regression to correlate gene expression with bioaccumulation data in seals, cormorant, fish, and mussels (Cheung et al., 2001; Ferreira et al., 2005; Hirakawa et al., 2007; Nakayama et al., 2006, 2008; Poynton et al., 2014). We proposed that this method could help identify biomarkers of exposure to the most prevalent contaminants in Boston Harbor.

We found several genes that were significantly correlated with elevated concentrations of PCBs and PAHs in the deployed mussels. Interestingly, genes that were found to correlate with PAHs and PCBs had functions that were similar to the enrichment analysis at IH. This is expected as IH was the most contaminated site and was likely driving the correlation analysis. For example, in the correlation analysis genes were found to be involved in signal transduction (calmodulin, Serine threonine-kinase SIK3, Integrin-alpha FG-GAP repeat-containing partial, Rab6), cilium assembly (cytoplasmic dynein 2 heavy chain 1), cell death (calmodulin), and oxidation reduction process (sulfide:quinone mitochondrial).

The correlation analysis identified several candidate biomarkers for PAHs and PCBs. These genes were involved in stress response (sirtuins), vesicular traffic (Rab6), apoptosis (calmodulin), endocytosis (Macrophage mannose receptor 1, Tyrosine- phosphatase non-receptor type 6), tumor suppressing (Serine threonine kinase SIK3) and cell division (cyclin-related FAM58A) (Table 3). However, several well characterized biomarkers for PAHs and PCBs, including DNA damage, oxidative stress and detoxification related genes, were not present in our data set (Cajaraville et al., 2000; Cheung et al., 2001; Ferreira et al., 2005). It is possible that concentrations may not have been high enough to activate these pathways, or there may have been environmental factors present at IH that interfered with the results. Follow-up laboratory studies that examine the expression of the identified correlation genes across contaminants may help determine if the genes were responding specifically to PAHs and PCBs or other variables.

4.3. Did transcriptomic responses in deployed mussels suggest adverse effects?

In addition to identifying exposure biomarkers, we were also interested in the overall transcriptomic profiles, as it may provide insight into the health of the mussels deployed at each site. By deploying caged mussels from a cleaner reference population, mussels can be used to determine the extent of contaminant bioaccumulation across different locations. The MWRA assessed accumulation levels in mussel tissues by comparing to threshold levels that are based on FDA limits. The tissue concentration at 60 days were below MWRA threshold warning and caution levels at all locations and contaminants (Hall et al., 2013). Although these contaminants are below set standards, these standards are based on limits for human consumption and do not consider harm to the mussels and wildlife. Therefore, concentration levels may still be at levels to impact marine life or there may be other sub-lethal impacts occurring that were not accounted for in previous studies.

The IH mussels had the highest concentrations of accumulated contaminants (PCBs, DDT, chlordanes, PAHs) compared to any of the other analyzed sites. The gene expression results at IH supported evidence of toxicant exposure as response to drug was a significantly enriched up-regulated GO term. Although not the most polluted, DI had

the most distinct transcriptomic profile compared to the other sites based on the number of DEGs and enrichment analysis. These results at DI may be explained by non-contaminant biotic and abiotic factors because the enriched up-regulated GO terms included external stimulus and response to nutrient levels. If there were changes in nutrients or external factors at DI, this could impact growth and gonadal development of deployed mussels. This was suggested by the up-regulation of GO terms of growth and down-regulation of anatomical structure morphogenesis, and embryo development. There were also many genes both up and down regulated at DI that mapped to the cell cycle which would be expected for mussels that were in a different developmental stage than the reference site. It is possible that external factors stimulated gonadal development in the mussels at DI altering their stage of gametogenesis in comparison to the ME reference site.

However, the transcriptomic data also suggested that DI mussels were showing signs of stress. Four enriched genes that were up-regulated were related to double-stranded break repair including structural maintenance of chromosomes protein 6- DNA polymerase alpha catalytic subunit, AP-5 complex subunit sigma-1, and protein MMS22. There were also 14 heat shock proteins differentially expressed at DI. The MWRA reported that flounder collected from DI had bent fin ray (22%) and fin erosions (44%) which are signs of environmental stress, disease, or sub-optimal water conditions (Hall et al., 2013; Nestler et al., 2013).

Although the OS site had the highest IBR score, there were less than 150 DEGs and only a few enriched GO terms at the OS compared to ME. This contrasts with the lab EDC exposure (Blalock et al., 2018) in which there were almost 2000 DEGs. The fewer number of significant genes is most likely due to the increased variability of the field conditions or the sample size used in the microarray analysis. In agreement with the C-BED assay IBR results, there was evidence of potential endocrine disruption at the OS site in the enrichment analysis. Genes mapped to cell division were up-regulated while response to endogenous stimulus was a down-regulated GO term including a receptor that binds to hormonal response elements (nuclear receptor subfamily 2 group c member 1). Past bioassessments conducted by the MWRA at OS also revealed potential system impairment, particularly in flounder. For example, fish caught at OS were substantially lower weight than historically recorded and had an increased incidence of blind side ulcers (Hall et al., 2013).

4.4. IBR as a transcriptomic tool for environmental monitoring

Monitoring programs such as “Mussel Watch” have relied on bivalves including blue mussels as indicators of seawater contamination for decades providing an indispensable dataset of the long-term trends of major contaminants (Melwani et al., 2014). Building off these programs, Galloway et al. (2006) proposed integrating biological responses into monitoring programs to gage marine ecosystem health, which may include ‘omics tools and molecular biomarkers (Veldhoen et al., 2012). However, several challenges remain for incorporating ‘omics into monitoring programs, including understanding the influence of non-contaminant environmental factors on gene expression and relating transcriptomic changes to adverse effects relevant for ecosystem health assessments (Bahamonde et al., 2016; Campos et al., 2018). To tackle these challenges, many have proposed developing more specific biomarkers that are linked to adverse effects through AOPs (Bahamonde et al., 2016; Fairbrother et al., 2019). However, the question remains: what is the best method to communicate changes in gene expression into simple metrics and visualizations that provide a straightforward indication of exposure or risk (Shaw et al., 2011; Campos et al., 2018)? Here we show the value of incorporating gene expression biomarkers into simple star plots created using the IBR. Part of the power of our approach is that the biomarkers were previously selected for their potential to act on an AOP of endocrine disruption in blue mussels (Blalock et al., 2018).

A number of previous studies have used the IBR to summarize and visualize the effects of multiple transcriptomic biomarkers. These studies include laboratory exposures to known contaminants and mixtures (Beaumelle et al., 2017; Li et al., 2018; Qiu et al., 2018) and in field studies (Pain-Devin et al., 2014; Kim and Jung, 2016) using a multilevel IBR approach (Kim et al., 2014). In most cases, the IBR has been used to prioritize the risk potential of different treatments or rank the environmental health of different sites. Because the value of the IBR depends upon the responses of each the biomarkers, it is essential that these genes are selected carefully and are relevant to the adverse effects or exposures you are trying to quantify. Different studies have approached biomarker selection differently. For example, Liu et al. (2019), used regression analysis to determine which biomarkers best correlated with polybrominated diphenyl ether (PBDE) exposure, while others have focused on single biological pathways and chose different genes within that pathway to indicate the level of perturbation to that biological process (Qiu et al., 2018). When developing the C-BED assay, we used both a targeted and a systems biology approach, and selected a set of biomarkers representing key regulatory hubs of steroidogenesis and endocrine response as well as genes representative of adverse effects. In the present study, we incorporated these biomarkers into the IBR allowing us to distinguish between sites where only one or a few of the genes were responding in a non-specific manner, and sites where the endocrine system of the mussels was being disrupted by probable EDC exposure. In addition, sites with higher potential EDC exposure from either wastewater outfall or CSOs elicited a greater IBR score, highlighting the potential for C-BED assay biomarkers to detect exposure from multiple sources.

The IBR is a powerful visualization and communication tool and is one of the few, easy to understand methods for combining multiple biomarker results into a single metric (Beliaeff and Burgeot, 2002). Improvements to the calculation of the IBR have been made since its introduction, but other limitations remain. Devin et al. (2014) recognized that the IBR score is dependent on the physical arrangement of the biomarkers in star diagrams, which led to its misuse in several studies. They proposed a method used here based on the median IBR score following all possible permutations. An alternative approach was applied by Kim et al. (2014) which averages biomarker responses at the molecular, biochemical, and physiological level before determining IBR scores. Outside of the original technical challenges, the IBR approach is still limited to ranking treatments or sites within a single study because the biomarker responses are normalized across the conditions. This also makes the IBR sensitive to treatment or site outliers as one high IBR score can skew the results at the other locations. In our study, we used an in-laboratory exposure control to compare to our field exposures. Sites with IBR scores above our laboratory controls were considered exposed to EDCs and likely impaired. Another limitation we have noticed in the application of the IBR is that it currently does not account for variability of individual genes, making it difficult to distinguish and filter true transcriptomic responses from background variability. New developments in the IBR approach could focus on making IBR scores more transferable across studies and incorporating statistical testing into the metric. Finally, to make the IBR method most useful to managers and regulators, we need a way to translate IBR scores into levels of risk (low, medium, high) for environmental health evaluations.

5. Conclusion

Transcriptomic biomarkers can help improve and build off existing biomonitoring programs as they are sensitive, cost effective, and demonstrate that contaminants have entered organisms and are eliciting an adverse effect. This study illustrates several approaches for using gene expression data to help target different classes of contaminants in field monitoring. First, multiple regression analysis showed potential in identifying new biomarkers for PAH and PCB exposure illustrating how global technologies such as omics’ can help develop specific gene fingerprints that respond to contaminants and allow faster identification

of candidate biomarkers. Testing these genes in laboratory exposures will help validate these biomarkers for future use. Secondly, the enrichment analysis suggested that mussels at IH and possibly DI had impacts to stress and detoxification which is expected for mussels at the most contaminated locations. Finally, the C-BED biomarkers and IBR analysis demonstrated that sites with higher potential EDC exposure from either wastewater outfalls or CSOs elicited a greater IBR score, highlighting the potential for C-BED assay biomarkers to detect exposure from multiple sources. Our success with the IBR approach relied on selecting EDC biomarkers nested within a putative AOP, including key regulators of signaling pathways, steroidogenesis, and genes representative of adverse effects (Blalock et al., 2018). Overall, this study can be used as an example for how multiple transcriptomic methods can be used to assess the impacts of pollution in marine environments.

Author contributions

BJB, WER, and HCP conceived of the study, BJB designed the study, BJB and HCP carried out the experimental study, BJB analyzed the results, BJB, WER, and HCP wrote the paper.

Declaration of competing interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.105397.

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