



First insight into the development of a new transcriptomic tool in French Corsica harbors

Marion Pillet, K. Garrett Evensen, Michel Marengo, Pierre Lejeune, Helen Poynton, H  l  ne Thomas

► To cite this version:

Marion Pillet, K. Garrett Evensen, Michel Marengo, Pierre Lejeune, Helen Poynton, et al.. First insight into the development of a new transcriptomic tool in French Corsica harbors. *Marine Pollution Bulletin*, 2022, 184, pp.114173. 10.1016/j.marpolbul.2022.114173 . hal-03894103

HAL Id: hal-03894103

<https://hal.science/hal-03894103>

Submitted on 30 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin  e au d  p  t et    la diffusion de documents scientifiques de niveau recherche, publi  s ou non,   manant des   tablissements d'enseignement et de recherche fran  ais ou   trangers, des laboratoires publics ou priv  s.

First insight into the development of a new transcriptomic tool in French Corsica harbors

Marion Pillet^{1,3†}, K. Garrett Evensen^{2†}, Michel Marengo¹, Pierre Lejeune¹, Helen C. Poynton²
& H       Thomas³

¹STation de REcherches Sous-marines et Océanographiques, Punta Revellata, 20260 Calvi,
France

²School for the Environment, University of Massachusetts, 100 William T. Morrissey Blvd.,
Boston, MA 02125, United States

³Littoral ENvironnement et Sociétés (UMR7266 CNRS), La Rochelle Université, 2 rue Olympe de Gouges, 17000 La Rochelle, France

[†] These authors contributed equally to this work

*Corresponding author at: marion.pillet@univ-lr.fr

Abstract

Coastal harbor areas are subjected to a myriad of pollution sources with largely unknown effects. Such complex chemical mixtures are difficult to monitor but transcriptomics is a promising approach for such biomonitoring. The present study was designed to verify the use of the Coastal Biosensor for Endocrine Disruption (C-BED) assay, previously developed to detect emerging contaminants and their effects on *Mytilus edulis*, on another mussel species, *Mytilus galloprovincialis*. Mussels were caged on St-Florent harbor (contaminated) and on Revellata Bay (reference) for three months. A classical multibiomarkers approach was coupled to the C-BED assay. The results of both approaches were analysed using the Integrated Biomarkers Responses (IBR) and compared to each other. Both approaches demonstrated a higher contamination and probable endocrine disruption of mussels in St-Florent, compared to the reference station. These results confirm that the C-BED assay provides an innovative method to expands our ability to detect emerging contaminants.

Keywords: chronic pollution, emergent contaminant, C-BED, biomonitoring, *M. galloprovincialis*, multibiomarkers.

Introduction

Coastal harbor areas are susceptible chronic and diffuse marine pollution due to contaminant run-off from natural and anthropogenic sources (e.g. terrestrial discharges, anthropogenic contaminations) and estuarine characteristics (e.g., low hydrodynamism) (Islam and Tanaka, 2004). This pollution is a serious threat to the marine environment and recreational activities and could pose a subsequent risk to human health (Maisano et al., 2017). Recently, the presence of legacy pollutants (trace elements, PAHs, PCBs) has declined within urban coastal systems, but emerging contaminants (e.g. pharmaceuticals, perfluorinated alkylated substances,

nanomaterials) are becoming more prominent even as their impacts are not fully understood (Maruya et al., 2014; Richardson, 2009). The concern about these compounds has increased due to endocrine disruptive effect and their potential to cause population level effects in wildlife at low exposure levels (Blalock et al., 2020). In addition, these chemicals are frequently found together in complex mixtures. Determining the impacts of emerging contaminants and how they interact with other chemical stressors to cause additive or synergistic effects is one of the major challenges in ecotoxicology today (Van den Brink et al., 2018).

In 2008, the European Commission approved the Marine Strategy Framework Directive (DCSMM, 2008), a legislation aimed at achieving or maintaining “Good Environmental Status” in EU waters. In North Corsica, to our knowledge, no study has been done within its harbor areas, although it is important to evaluate and monitor the quality of the waters and the ecosystem.

Marine biomonitoring consists in observing and assessing the natural or induced alterations of marine ecosystem and this evaluation can be measured at several levels, from the molecular, cellular, and individual levels to the populations up to whole biocenosis level (Bondaruk et al., 2015). Bivalves and especially mussels have been used as bioindicators for decades to investigate the toxicity of pollutants. The classical example of biomonitoring is the Mussel Watch concept, which was initiated in 1976, consisting in the surveillance of United States coastal regions for their pollutant contents (Farrington et al., 2016). The use of these bioindicators is particularly relevant from a biological point of view as the directly measured concentrations reflect the bioavailable fractions in the marine environment (Wang, 2016). Mussels are ideal for biomonitoring studies because they are filter feeders, are distributed worldwide, and have capacity to bioaccumulate diverse contaminants (Banni et al., 2016; Canesi et al., 2008; Ozkan et al., 2017; Strehse and Maser, 2020). They also provide several

biomarker responses, such as decreased antioxidant defences in the presence of pollution (Beyer et al., 2017).

Despite the sensitivity of conventional biomarkers for biomonitoring and risk assessment (Handy et al., 2012), these indicators may not respond to low level, chronic exposures to pollutants or exposure to emergent pollutants. More integrative tools, are needed that offer greater sensitivity and specificity across diverse pollutant classes (Gottschalk et al., 2013). Transcriptomic and other High Throughput Screening (HTS) approaches, are of great interest because they allow the screening of a large set of genes and the impact of pollutants on their expression (Châtel et al., 2018; Poynton and Vulpe, 2009).

In the present study, a classic multi-biomarker approach was coupled to an innovative transcriptomic approach that uses the biological responses of blue mussels to detect the additive impacts of complex contaminant mixtures found in urban coastal areas. The Coastal Biosensor for Endocrine Disruption (C-BED), was designed based the responses of *Mytilus edulis* exposed to estrogenic compounds in the lab, and was successfully validated by detecting endocrine disrupting compounds (EDCs) in field collected mussels in Northeastern U.S.A. (Blalock et al., 2018; Blalock et al., 2020). The assay uses the transcriptomic responses of a specific set of biomarker genes chosen because of their roles in endocrine pathways and sexual development, and their robust responses to endocrine disrupting stressors.

The goal of this paper is to translate this innovative method to a different mussel species, *M. galloprovincialis*, and to test the incorporation of a molecular tool in a classic biomonitoring in Mediterranean harbors of North Corsica.

Material and methods

Caged mussels (*M. galloprovincialis*) were deployed in St. Florent harbor (exposed site, North Corsica, N 42° 40' 50.362"; E 9° 17' 54.373") and in the Revellata Bay (control site, N 42° 34'

49.584"; E 8° 43' 31.511") for three months before sampling in September 2020 and January 2021. At the end of the exposure, 20 mussels were collected for subsequent classic biomarkers (n = 10) and molecular (C-BED, n = 10) analyses.

A. Classic biomarker approach

Individuals collected for the classic biomarkers were immediately placed in dry ice, shipped to the laboratory within 48 h and kept at -80°C until enzymatic analyses.

The digestive gland of mussel was dissected and ground in liquid nitrogen using a MM400 Retsch® (GmbH, Éragny, Luxemburg) mixer mill and homogenized on ice in phosphate buffered saline (PBS) solution (100 mM, pH 7.5) containing 0.1 % Triton X-100 and 1 mM methylene diamine tetra-acetic acid (EDTA) to avoid protein degradation. The samples were centrifuged, and supernatant was collected for the subsequent biomarkers of effect and protein analyses.

A multibiomarkers approach was used by testing superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione S-transferase (GST), pyruvate kinase (PK), acetylcholinesterase (AChE) and laccase-type phenoloxidase (LAC). SOD activity protocol is based on the inhibition of a superoxide-driven NADH oxidation and was measured following the rate of NADH oxidation at 340 nm (Paoletti et al., 1986). CAT activity was determined according to Aebi (1984) by measuring the rate of decomposition of hydrogen peroxide at 240 nm. GPx activity was assayed according to Paglia and Valentine (1967) as modified by Janssens et al. (2000) by monitoring the consumption of NADPH at 340 nm. GST activity was determined by measuring the production of GS-DNB at 340 nm (Habig et al., 1974). The activity of PK was determined by measuring the NADH consumption at 340 nm, according to Childress and Somero (1979) as modified by Bailey et al. (2005). AChE activity was assayed by recording the production of 5-thio-2-nitrobenzoate at 405 nm (Ellman et al., 1961). Finally,

the LAC activity was measured according to Luna-Acosta et al. (2010), following the oxidation of phenylenediamine at 420 nm. The total protein content was measured with the bicinchoninic acid protein assay kit (Sigma-Aldrich®) that is similar to the Lowry et al. (1951) procedure. All activities measurements were done on a SpectroStar Nano spectrophotometer (BMG labtech, Champigny-sur-Marne, France). Chemicals were obtained from Sigma-Aldrich®. All analyses were performed in duplicate using standard methods adapted for a microplate reader. For all enzymatic analysis, homogenates were diluted to obtain linear reaction slopes for a minimum of five minutes at 25 °C. Specific enzymatic activities were measured and expressed as U ($\mu\text{mol} \cdot \text{min}^{-1}$).mg⁻¹ of protein.

B. Transcriptomic approach

For the C-BED assay, mussel (n = 10 for each period) were immediately dissected and the digestive gland was kept in RNAlater. Total RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH), according to the manufacturer's protocol. Samples were then treated with DNase (New England Biolabs, Ipswich, MA) to remove any residual DNA. Samples were cleaned up using a NEB Monarch RNA Cleanup Kit (New England Biolabs) to purify the RNA samples. RNA was reverse transcribed to cDNA using EpiScript Reverse Transcriptase (Lucigen, Middleton, WI) (Blalock et al., 2018; Blalock et al., 2020; Hines et al., 2007).

The C-BED assay uses real time quantitative PCR (RT-qPCR) and consists of eleven genes: two sex marker genes (for sex identification: VERL, VCL), eight biomarker genes (Cav-1, StAR, MBTP, BCAS3, SR, ADX, GCL32E, Mat89bb), and one reference gene (18S) (Blalock et al., 2018; Blalock et al., 2020). *M. edulis* primers sequences from Blalock et al. (2018) were tested on the *M. galloprovincialis* samples and verified on an agarose gel. All primers, except VERL, produced a product. New primers for *M. galloprovincialis* VERL (GenBank:

VDI63802.1) were designed using Primer3 (v4.1.0) (F: GCT GTT GCG TTG CTA TCA CT
R: TGC AGA TGA CGC CTT TAT GC) and were tested and verified using qPCR.
All qPCR reactions were performed as described previously (Blalock et al., 2018) using
PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Waltham, MA). Standard
curves for all primers, including the newly designed *M. g.*, VERL primers, used had an r^2 of
0.99 and a primer efficiency between 95-105% (Blalock et al., 2018). The difference in
expression in VERL and VCL was used for sex identification of *M. galloprovincialis* mussels
(Blalock et al., 2018; Hines et al., 2007). A difference in Ct greater than 2.5 indicates the mussel
is a male, less than -2.5 is a female, and between -2.5 and 2.5 is unknown (i.e., sex cannot be
determined) (Blalock et al., 2018; Hines et al., 2007). Expression of the eight biomarker genes
were normalized to 18S expression. The C-BED assay was performed on all mussels including
those identified as male, female, or undetermined. This is in contrast with our previous study
using the C-BED assay, which limited assay responses to male mussels only (Blalock et al.,
2018). We have decided to extend the assay to all mussels because (1) there was limited
difference between the IBR score of males vs females in this study (see supplement) and (2) in
the presence of endocrine disrupting compounds, male mussels may become phenotypically
female and be identified as female mussels (Evensen in prep). Therefore, focusing only on
mussels identified as male following an exposure is not including the responses of all
genetically male mussels.

C. Statistics

Data were compiled into the Integrated Biomarker Response (IBR). IBR was developed by
(Beliaeff and Burgeot, 2002). The IBR was described to be useful in environmental quality
assessment to evaluate the impact of environmental conditions on the health of organisms and
their physiological status (Serafim et al., 2012) and was shown to be useful to distinguish among

sites located along a stress gradient, while individual biomarkers were not able to do so (Bertrand et al., 2018). Devin et al. (2014) adjusted the IBR calculation by determining every permutation of the IBR based on different positions of the biomarkers and calculating the median IBR value. The median IBR value was calculated for both sites at both time points. On these data, normality and homogeneity of variances were verified by Shapiro and Levene tests and data. As these conditions were not found and no data transformations was possible, non-parametric Kruskal-Wallis tests, were performed to analyse the effect of treatment, followed by Mann-Whitney Wilcoxon post-hoc test when a significant effect was found.

Results

Mussel sex identification was performed to evaluate for skewed sex ratios. There was no significantly skewed sex ratio at Revellata Bay at either time points or at St. Florent January 2021. There was a significantly skew sex ratio ($p < 0.05$) at St. Florent in September 2021 (**Figure 1**). At St. Florent in September, there was only one male out of the ten sampled mussels; the remaining mussels were all female.

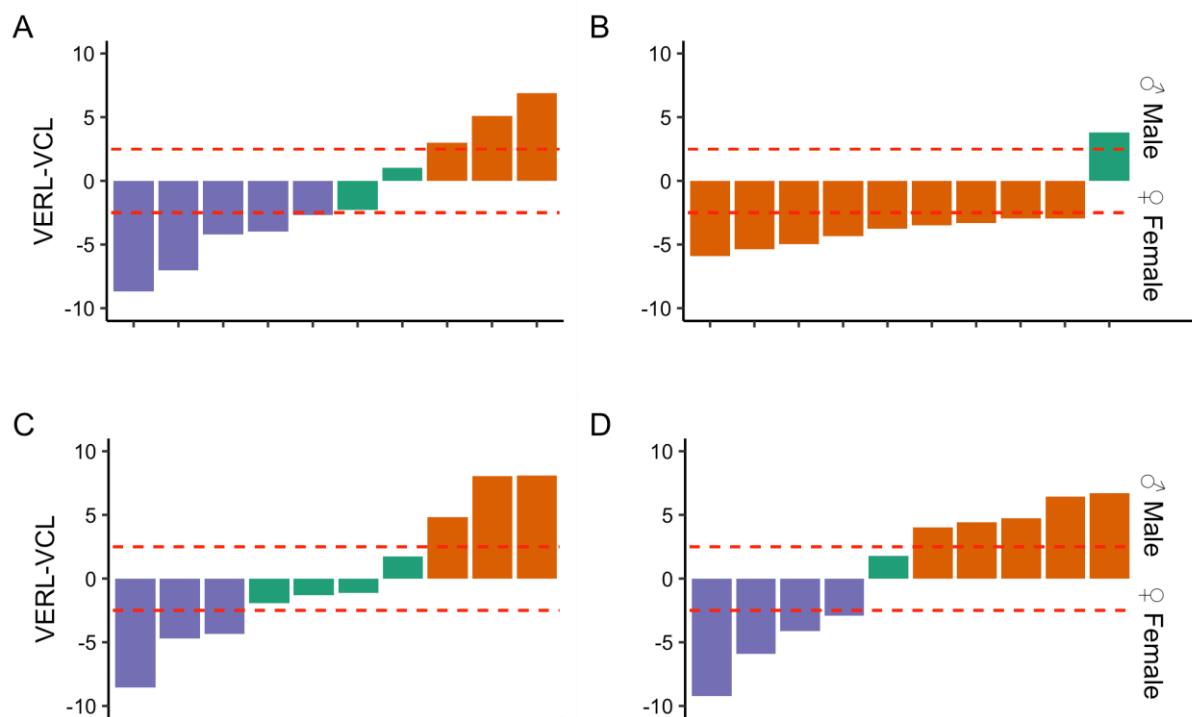


Figure 1: Sex identification results at Revellata Bay and St. Florent. Bars greater than 2.5 are male, and bars less than -2.5 are female. Bars that are between -2.5 and 2.5 indicate mussels whose sex could not be determined. A) Revellata Bay, September 2020. B) Revellata Bay, January 2021. C) St Florent, September 2020. D) St. Florent, January 2021.

The classical biomarkers showed higher IBR values (figure 2) at St-Florent (median = 5.37 in September and 4.62 in January) compared to Revellata Bay (median = 0.105 in September and 2.11 in January), and a greater difference in the summer.

The relative importance of each biomarker varies according to season and site: in St-Florent in September, all enzymes present relatively low coefficients, while the higher coefficients are observed for LAC, GPx, GST and PK in St-Florent in January and for CAT, ACHE and SOD in Revellata Bay at both seasons.

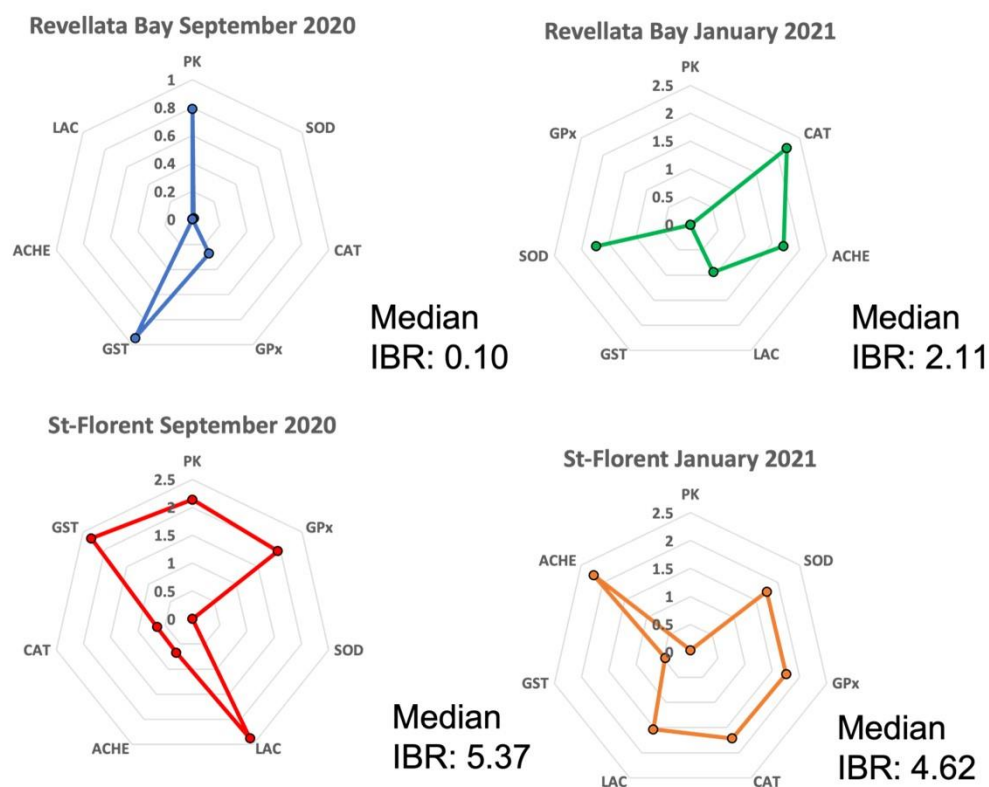


Figure 2: Median IBR values calculated using data from the classical biomonitoring data

Similarly to the classical biomarkers, the C-BED assay showed higher IBR values at St. Florent compared with Revellata Bay (**Figure 3**). At both time points, the IBR values at St. Florent were higher than Revellata Bay. In September 2020 and January 2021, Revellata Bay had IBR values less than 1, while St-Florent had IBR values greater than 1, reaching 8.76 in September 2020 (**Figure 3**). At both sites, the IBR values for January were lower than those for September (**Figure 3**). Based on the IBR values, we ranked the sites and time points based on risk of endocrine disruption: Revellata Bay January 2021 < Revellata Bay September 2020 < St. Florent January 2021 < St. Florent September 2020.

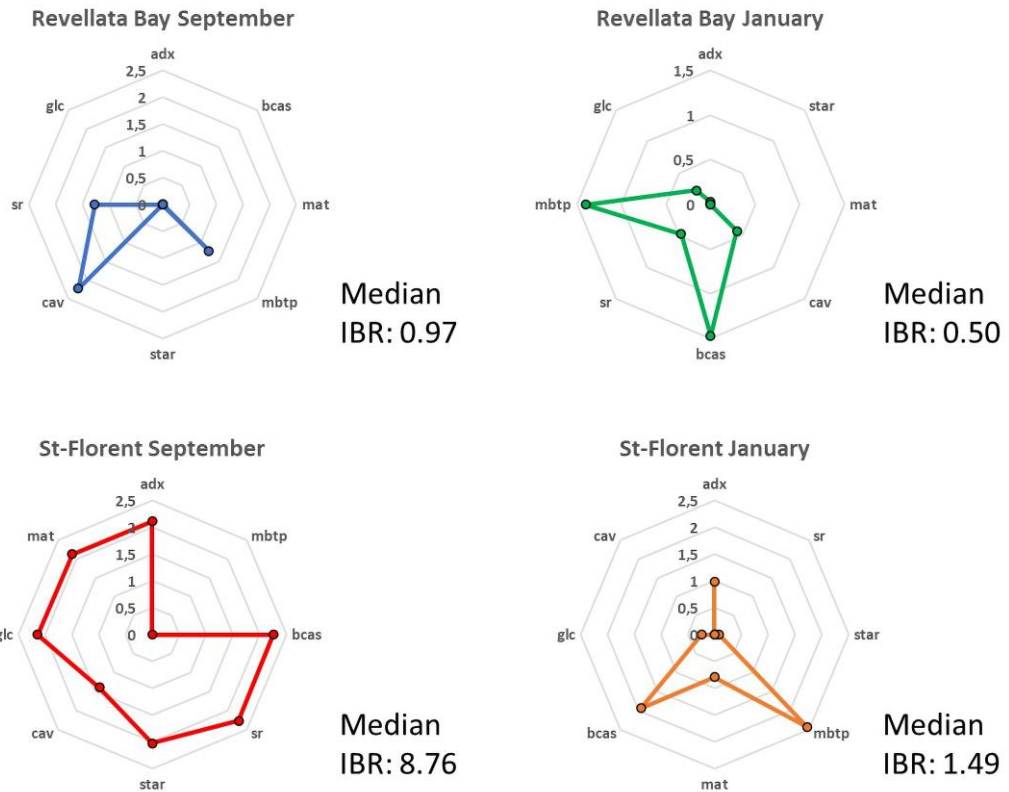


Figure 3: Median IBR values for Revellata Bay (September 2020, January 2021) and St Florent (September 2020, January 2021) for the C-BED Assay. The position of the biomarkers is adjusted for each condition to reach the median IBR value

Discussion

Mussels at St. Florent are exposed to a complex mixture

The C-BED assay and the other biomarkers indicate that *M. galloprovincialis* at St. Florent are exposed to a complex mixture of contaminants, including EDCs. The higher classic biomarker IBR values at St. Florent compared to Revellata Bay (**Figure 2**) indicate there are contaminants in the water, causing a stress response in the St. Florent mussels. These results confirm observations described in previous environmental biomonitoring studies. St-Florent is usually described as under moderate anthropogenic pressure (Gobert et al., 2009) or even as a highly contaminated site (Ternengo et al., 2018). The contamination is usually ascribed to high levels

of metallic trace elements such as Co, Cr, Fe or Ni, linked to waste from the former Canari asbestos mine, closed in 1965, located at the northwest of the Gulf of St-Florent (Andral et al., 2004; Luy et al., 2012; Ternengo et al., 2018). However, there is little data available on pollution levels in this region, especially for organic contaminants.

*C-BED assay is translatable across *Mytilus* species*

Here, we adapted the C-BED assay, which was originally developed in *M. edulis* (Blalock et al., 2018), to the closely related *M. galloprovincialis*. This assay is used as an early indicator for possible developmental and reproductive impairments. There was a higher median IBR value at both time points at St. Florent (Sept: 8.79, Jan: 1.49) than at Revellata Bay (**Figure 3**). The results of the C-BED assay indicate that mussels at St. Florent are responding to EDCs. Furthermore, the significantly feminized sex ratio ($p < 0.05$) at Revellata Bay in September (**Figure 3C**), during the same sampling period as the highest IBR values, strongly suggests that endocrine disruption is occurring in these mussels. In addition, the difference in IBR values at the impacted and reference site show that this assay can be applied to *M. galloprovincialis*. The *Mytilus* species complex are found globally, from South America to the North Atlantic to the Pacific (Oyarzún et al., 2021; Wilson et al., 2018). With the success of application of the C-BED assay to *M. galloprovincialis*, this assay can be utilized in other *Mytilus* species.

Benefits of a multi-biomarker approach

Using IBR values from both approaches indicates mussels are exposed to a variety of contaminants including contaminants that elicit classic stress responses and those that cause endocrine disruption. Interestingly, both approaches indicated that contamination levels and effects are more severe during the summer. These effects might be due to the higher touristic activity in summer or to the elevated temperature for example. A single compound at a low

concentration may not produce a detectable adverse effect in an organism. However, many contaminants at low concentrations may cause additive impacts, resulting in a detectable effect (Giuliani et al., 2013; Strömgren, 1986; Widdows and Donkin, 1992). Adding a transcriptomic assay to biomonitoring can provide vital information because gene expression changes are highly responsive and sensitive (Aymoz et al., 2018; Bendjilali et al., 2017). Furthermore, utilizing multiple assays and IBR values provides greater insight to possible exposures and stress responses organisms may be experiencing (Beliaeff and Burgeot, 2002; Devin et al., 2014; Giuliani et al., 2013; Kim et al., 2014).

Conclusion

Here we demonstrated that the C-BED assay is translatable to a new *Mytilus* species, broadening its application and that its inclusion within a multi-biomarker monitoring program provides insight into the chemicals present and causing biological effects within urban harbors. Determining the impacts of emerging contaminants and how they interact with other chemical stressors to cause additive or synergistic effects is one of the major challenges in ecotoxicology today. The global application of the C-BED assay addresses this challenge and will significantly enhance our ability to predict environmental impacts of contamination found in our urban coastal ecosystems and harbors. Through this, we anticipate the development of a transformative tool for coastal environmental assessment into management decisions for coastal areas.

Acknowledgment

The authors are grateful to Mr. B. Pantalacci, manager of the shellfish farm *SARL Etang de Diane*, and his employees who provided mussels.

Authors Contributions

Marion Pillet: conceptualization, methodology, validation, data collection, formal analyses, investigation, writing.

K. Garrett Evensen: methodology, validation, data collection, formal analyses, investigation, writing.

Michel Marengo: conceptualization, methodology, supervision, project administration, funding acquisition.

Pierre Lejeune: conceptualization, funding acquisition.

Helen C. Poynton: conceptualization, supervision, funding acquisition.

Hélène Thomas: supervision, project administration, funding acquisition.

Funding

This work was supported by the “**QUALity of the marine environment in the Mediterranean POrt areas**” (QUAMPO) project, funded by the “Fonds Européen pour les Affaires Maritimes et la Pêche” (FEAMP) - **European Maritime and Fisheries Fund (EMFF)** [grant number **PFEA800219DM0940001, 2019**].

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

The authors have no competing interests to declare that are relevant to the content of this article.

Ethical Approval

This study does not involve human participants or vertebrate animals.

Bibliography

- Aebi, H., 1984. Catalase in vitro, Methods Enzymol. Academic Press, pp. 121-126.
- Andral, B., Stanisiere, J.Y., Sauzade, D., Damier, E., Thebault, H., Galgani, F., Boissery, P., 2004. Monitoring chemical contamination levels in the Mediterranean based on the use of mussel caging. Mar. Pollut. Bull. 49, 704-712.
- Aymoz, D., Solé, C., Pierre, J.-J., Schmitt, M., de Nadal, E., Posas, F., Pelet, S., 2018. Timing of gene expression in a cell-fate decision system. Mol. Syst. Biol. 14, e8024-e8024.
- Bailey, D.M., Genard, B., Collins, M.A., Rees, J.-F., Unsworth, S.K., Battle, E.J.V., Bagley, P.M., Jamieson, A.J., Priede, I.G., 2005. High swimming and metabolic activity in the deep-sea eel *Synaphobranchus kaupii* revealed by integrated in situ and in vitro measurements. Physiol. Biochem. Zool. 78, 335-346.
- Banni, M., Sforzini, S., Balbi, T., Corsi, I., Viarengo, A., Canesi, L., 2016. Combined effects of n-TiO₂ and 2,3,7,8-TCDD in *Mytilus galloprovincialis* digestive gland: A transcriptomic and immunohistochemical study. Environ. Res. 145, 135-144.
- Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: A useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21, 1316-1322.
- Bendjilali, N., MacLeon, S., Kalra, G., Willis, S.D., Hossian, A.K.M.N., Avery, E., Wojtowicz, O., Hickman, M.J., 2017. Time-Course Analysis of Gene Expression During the *Saccharomyces cerevisiae* Hypoxic Response. G3: Genes, Genomes, Genetics 7, 221-231.
- Bertrand, L., Monferrán, M.V., Mouneyrac, C., Amé, M.V., 2018. Native crustacean species as a bioindicator of freshwater ecosystem pollution: A multivariate and integrative study of multi-biomarker response in active river monitoring. Chemosphere 206, 265-277.
- Beyer, J., Green, N.W., Brooks, S., Allan, I.J., Ruus, A., Gomes, T., Bråte, I.L.N., Schøyen, M., 2017. Blue mussels (*Mytilus edulis* spp.) as sentinel organisms in coastal pollution monitoring: A review. Mar. Environ. Res. 130, 338-365.

323 Blalock, B.J., Robinson, W.E., Loguinov, A., Vulpe, C.D., Krick, K.S., Poynton, H.C., 2018.
 324 Transcriptomic and Network Analyses Reveal Mechanistic-Based Biomarkers of Endocrine
 325 Disruption in the Marine Mussel, *Mytilus edulis*. Environ. Sci. Technol. 52, 9419-9430.

326 Blalock, B.J., Robinson, W.E., Poynton, H.C., 2020. Assessing legacy and endocrine disrupting
 327 pollutants in Boston Harbor with transcriptomic biomarkers. Aquat. Toxicol. 220, 105397.

328 Bondaruk, J., Janson, E., Wysocka, M., Chałupnik, S., 2015. Identification of hazards for water
 329 environment in the upper Silesian Coal Basin caused by the discharge of salt mine water
 330 containing particularly harmful substances and radionuclides. Journal of Sustainable Mining
 331 14, 179-187.

332 Canesi, L., Borghi, C., Ciacci, C., Fabbri, R., Lorusso, L., Vergani, L., Marcomini, A., Poiana,
 333 G., 2008. Short-term effects of environmentally relevant concentrations of EDC mixtures on
 334 *Mytilus galloprovincialis* digestive gland. Aquat. Toxicol. 87, 272-279.

335 Châtel, A., Lièvre, C., Barrick, A., Bruneau, M., Mouneyrac, C., 2018. Transcriptomic
 336 approach: A promising tool for rapid screening nanomaterial-mediated toxicity in the marine
 337 bivalve *Mytilus edulis* - Application to copper oxide nanoparticles. Comparative Biochemistry
 338 and Physiology Part C: Toxicology & Pharmacology 205, 26-33.

339 Childress, J.J., Somero, G.N., 1979. Depth-related enzymic activities in muscle, brain and heart
 340 of deep-living pelagic marine teleosts. Mar. Biol. 52, 273-283.

341 DCSMM, 2008. Directive 2008/56/EC of 17 June 2008 establishing a framework for
 342 community action in the field of marine environmental policy (Marine Strategy Framework
 343 Directive), Official Journal of the European Union. European Commission.

344 Devin, S., Burgeot, T., Giambérini, L., Minguez, L., Pain-Devin, S., 2014. The integrated
 345 biomarker response revisited: optimization to avoid misuse. Environmental Science and
 346 Pollution Research 21, 2448-2454.

347 Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid
 348 colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88-95.
 349 Farrington, J.W., Tripp, B.W., Tanabe, S., Subramanian, A., Sericano, J.L., Wade, T.L., Knap,
 350 A.H., 2016. Edward D. Goldberg's proposal of "the Mussel Watch": Reflections after 40 years.
 351 *Mar. Pollut. Bull.* 110, 501-510.
 352 Giuliani, M.E., Benedetti, M., Arukwe, A., Regoli, F., 2013. Transcriptional and catalytic
 353 responses of antioxidant and biotransformation pathways in mussels, *Mytilus galloprovincialis*,
 354 exposed to chemical mixtures. *Aquat. Toxicol.* 134-135, 120-127.
 355 Gobert, S., Sartoretto, S., Rico-Raimondino, V., Andral, B., Chery, A., Lejeune, P., Boissery,
 356 P., 2009. Assessment of the ecological status of Mediterranean French coastal waters as
 357 required by the Water Framework Directive using the *Posidonia oceanica* Rapid Easy Index:
 358 PREI. *Mar. Pollut. Bull.* 58, 1727-1733.
 359 Gottschalk, F., Kost, E., Nowack, B., 2013. Engineered nanomaterials in water and soils: A risk
 360 quantification based on probabilistic exposure and effect modeling. *Environ. Toxicol. Chem.*
 361 32, 1278–1287.
 362 Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic
 363 step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130-7139.
 364 Handy, R.D., Cornelis, G., Fernandes, T., Tsyusko, O., Decho, A., Sabo-Attwood, T., Metcalfe,
 365 C., Steevens, J.A., Klaine, S.J., Koelmans, A.A., Horne, N., 2012. Ecotoxicity test methods for
 366 engineered nanomaterials: Practical experiences and recommendations from the bench.
 367 *Environ. Toxicol. Chem.* 31, 15–31.
 368 Hines, A., Yeung, W.H., Craft, J., Brown, M., Kennedy, J., Bignell, J., Stentiford, G.D., Viant,
 369 M.R., 2007. Comparison of histological, genetic, metabolomics, and lipid-based methods for
 370 sex determination in marine mussels. *Anal. Biochem.* 369, 175-186.

371 Islam, S.M., Tanaka, M., 2004. Impacts of pollution on coastal and marine ecosystems
 372 including coastal and marine fisheries and approach for management: a review and synthesis.
 373 Mar. Pollut. Bull. 48, 624-649.

374 Janssens, B.J., Childress, J.J., Baguet, F., Rees, J.F., 2000. Reduced enzymatic antioxidative
 375 defense in deep-sea fish. J. Exp. Biol. 203, 3717-3725.

376 Kim, W.-K., Lee, S.-K., Park, J.-W., Choi, K., Cargo, J., Schlenk, D., Jung, J., 2014. Integration
 377 of multi-level biomarker responses to cadmium and benzo[k]fluoranthene in the pale chub
 378 (*Zacco platypus*). Ecotoxicol. Environ. Saf. 110, 121-128.

379 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the
 380 Folin phenol reagent. J. Biol. Chem. 193, 265-275.

381 Luna-Acosta, A., Rosenfeld, E., Amari, M., Fruitier-Arnaudin, I., Bustamante, P., Thomas-
 382 Guyon, H., 2010. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. Fish
 383 Shellfish Immunol. 28, 719-726.

384 Luy, N., Gobert, S., Sartoretto, S., Biondo, R., Bouqueneau, J.-M., Richir, J., 2012. Chemical
 385 contamination along the Mediterranean French coast using *Posidonia oceanica* (L.) Delile
 386 above-ground tissues: a multiple trace element study. Ecol. Indicators 18, 269-277.

387 Maisano, M., Cappello, T., Natalotto, A., Vitale, V., Parrino, V., Giannetto, A., Oliva, S.,
 388 Mancini, G., Cappello, S., Mauceri, A., Fasulo, S., 2017. Effects of petrochemical
 389 contamination on caged marine mussels using a multi-biomarker approach: Histological
 390 changes, neurotoxicity and hypoxic stress. Mar. Environ. Res. 128, 114-123.

391 Maruya, K.A., Dodder, N.G., Weisberg, S.B., Gregorio, D., Bishop, J.S., Klosterhaus, S.,
 392 Alvarez, D.A., Furlong, E.T., Bricker, S., Kimbrough, K.L., Lauenstein, G.G., 2014. The
 393 Mussel Watch California pilot study on contaminants of emerging concern (CECs): Synthesis
 394 and next steps. Mar. Pollut. Bull. 81, 355-363.

395 Oyarzún, P.A., Toro, J.E., Nuñez, J.J., Suárez-Villota, E.Y., Gardner, J.P.A., 2021. Blue
 396 mussels of the *Mytilus edulis* species complex from South America: The application of species
 397 delimitation models to DNA sequence variation. PLOS ONE 16, e0256961.
 398 Ozkan, D., Dagdeviren, M., Katalay, S., Guner, A., Yavaşoğlu, N.Ü.K., 2017. Multi-biomarker
 399 responses after exposure to pollution in the Mediterranean mussels (*Mytilus galloprovincialis*
 400 L.) in the Aegean coast of Turkey. Bull. Environ. Contam. Toxicol. 98, 46-52.
 401 Paglia, D.E., Valentine, W.N., 1967. Studies on the quantitative and qualitative characterization
 402 of erythrocyte glutathione peroxidase. The Journal of Laboratory and Clinical Medicine 70,
 403 158-169.
 404 Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A., 1986. A sensitive spectrophotometric
 405 method for the determination of superoxide dismutase activity in tissue extracts. Anal.
 406 Biochem. 154, 536-541.
 407 Poynton, H.C., Vulpe, C.D., 2009. Ecotoxicogenomics: Emerging Technologies for Emerging
 408 Contaminants. Journal of the American Resources Association 45, 83-96.
 409 Richardson, S.D., 2009. Water Analysis: Emerging Contaminants and Current Issues.
 410 Analytical Chemistry 81, 4645–4677.
 411 Serafim, A., Company, R., Lopes, B., Fonseca, V.F., França, S., Vasconcelos, R.P., Bebianno,
 412 M.J., Cabral, H.N., 2012. Application of an integrated biomarker response index (IBR) to assess
 413 temporal variation of environmental quality in two Portuguese aquatic systems. Ecol. Indicators
 414 19, 215-225.
 415 Strehse, J.S., Maser, E., 2020. Marine bivalves as bioindicators for environmental pollutants
 416 with focus on dumped munitions in the sea: A review. Mar. Environ. Res. 158, 105006.
 417 Strömberg, T., 1986. The combined effect of copper and hydrocarbons on the length growth of
 418 *Mytilus edulis*. Mar. Environ. Res. 19, 251-258.

419 Ternengo, S., Marengo, M., El Idrissi, O., Yepka, J., Pasqualini, V., Gobert, S., 2018. Spatial
420 variations in trace element concentrations of the sea urchin, *Paracentrotus lividus*, a first
421 reference study in the Mediterranean Sea. Mar. Pollut. Bull. 129, 293-298.

422 Van den Brink, P.J., Boxall, A.B.A., Maltby, L., Brooks, B.W., Rudd, M.A., Backhaus, T.,
423 Spurgeon, D., Verougstraete, V., Ajao, C., Ankley, G.T., Apitz, S.E., Arnold, K., Brodin, T.,
424 Cañedo-Argüelles, M., Chapman, J., Corrales, J., Coutellec, M.-A., Fernandes, T.F., Fick, J.,
425 Ford, A.T., Giménez Papiol, G., Groh, K.J., Hutchinson, T.H., Kruger, H., Kukkonen, J.V.K.,
426 Loutseti, S., Marshall, S., Muir, D., Ortiz-Santaliestra, M.E., Paul, K.B., Rico, A., Rodea-
427 Palomares, I., Römbke, J., Rydberg, T., Segner, H., Smit, M., van Gestel, C.A.M., Vighi, M.,
428 Werner, I., Zimmer, E.I., van Wensem, J., 2018. Toward sustainable environmental quality:
429 Priority research questions for Europe. Environ. Toxicol. Chem. 37, 2281-2295.

430 Wang, W.X., 2016. Chapter 4 - Bioaccumulation and Biomonitoring, in: Blasco, J., Chapman,
431 P.M., Campana, O., Hampel, M. (Eds.), Marine Ecotoxicology. Academic Press, pp. 99-119.

432 Widdows, J., Donkin, P., 1992. Mussels and environmental contaminants : bioaccumulation
433 and physiological aspects, in: Gosling, E. (Ed.), The Mussel *Mytilus*: Ecology, Physiology,
434 Genetics and Culture. Elsevier, Amsterdam, pp. 383-424.

435 Wilson, J., Matejusova, I., McIntosh, R.E., Carboni, S., Bekaert, M., 2018. New diagnostic SNP
436 molecular markers for the *Mytilus* species complex. PLOS ONE 13, e0200654.

437

438

439

440 List of captions:

441 Figure 4: Sex identification results at Revellata Bay and St. Florent. Bars greater than 2.5 are
442 male, and bars less than -2.5 are female. Bars that are between -2.5 and 2.5 indicate mussels
443 whose sex could not be determined. A) Revellata Bay, September 2020. B) Revellata Bay,
444 January 2021. C) St Florent, September 2020. D) St. Florent, January 2021.

445 Figure 5: Median IBR values calculated using data from the classical biomonitoring data

446 Figure 6: Median IBR values for Revellata Bay (September 2020, January 2021) and St Florent
447 (September 2020, January 2021) for the C-BED Assay. The position of the biomarkers is
448 adjusted for each condition to reach the median IBR value

449

450

451

452