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Review

Environmental epigenetics: A promising venue for developing next-generation pollution biomonitoring tools in marine invertebrates

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ABSTRACT

Environmental epigenetics investigates the cause-effect relationships between specific environmental factors and the subsequent epigenetic modifications triggering adaptive responses in the cell. Given the dynamic and potentially reversible nature of the different types of epigenetic marks, environmental epigenetics constitutes a promising venue for developing fast and sensible biomonitoring programs. Indeed, several epigenetic biomarkers have been successfully developed and applied in traditional model organisms (e.g., human and mouse). Nevertheless, the lack of epigenetic knowledge in other ecologically and environmentally relevant organisms has hampered the application of these tools in a broader range of ecosystems, most notably in the marine environment. Fortunately, that scenario is now changing thanks to the growing availability of complete reference genome sequences along with the development of high-throughput DNA sequencing and bioinformatic methods. Altogether, these resources make the epigenetic study of marine organisms (and more specifically marine invertebrates) a reality. By building on this knowledge, the present work provides a timely perspective highlighting the extraordinary potential of environmental epigenetic analyses as a promising source of rapid and sensible tools for pollution biomonitoring, using marine invertebrates as sentinel organisms. This strategy represents an innovative, groundbreaking approach, improving the conservation and management of natural resources in the oceans.

Contents

1. A framework for the epigenetic analysis of environmental responses

One of the most amazing features of the eukaryotic genetic material is its ability to be packed and organized within a tiny cell nucleus that can be up to 200,000 times smaller. This is possible

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thanks to the wrapping of the DNA molecule around chromosomal proteins (most notably histones), constituting a dynamic polymer organized in fundamental nucleosome subunits known as chromatin. Beyond structural considerations, chromatin also participates in the functional classification of the information contained in the genome (Allis et al., 2007), providing a framework for the study of epigenetics, defined as the heritable changes in gene expression resulting from modifications in chromatin structure, without involving changes in the genetic information stored in the DNA sequence (Allis et al., 2007).

Various mechanisms have the potential to encode epigenetic information including DNA methylation, the replacement of canonical histones by specialized histone variants, histone post-translational modifications (PTMs), non-coding RNAs, and transcription factor regulatory networks, among others (Kouzarides, 2007; Ptashne, 2007; Arya et al., 2010; Talbert and Henikoff, 2010; Mercer and Mattick, 2013). Although different in nature, all these mechanisms are able to trigger dynamic modifications of the chromatin structure in response to external stimuli (Talbert and Henikoff, 2014). However, while some of these modifications last for a few seconds before being rapidly reverted to a basal state (e.g., acetylation of histones allowing expression of genes specifically involved in DNA repair), others may persist in the chromatin of the same specific cell for decades [e.g., DNA methylation leading to gene silencing during the differentiation of neural stem cells (Williams et al., 2014)]. Furthermore, what it is truly amazing about these epigenetic marks is their ability to transcend across generations, constituting the basis for long-term adaptations [e.g., conserved DNA methylation imprinting in the germ line (Gapp et al., 2014; Heard and Martienssen, 2014), Fig. 1].

Overall, epigenetics constitutes the next frontier for understanding how mechanisms of temporal and spatial control of gene activity operate during adaptive responses to external stimuli (Holliday, 1990). In order to do so, it is fundamental to investigate not only the links between specific epigenetic marks and the subsequent modifications in chromatin structure and gene expression, but also the environmental factors leading to these epigenetic marks in the first place (Cortessis et al., 2012). That strategy constitutes the basis for environmental epigenetic analyses (Baccarelli and Bollati, 2009; Bollati and Baccarelli, 2010), providing information about the mechanisms by which different environmental factors influence phenotypic variation, both within individuals and across generations [(Cortessis et al., 2012; Talbert and Henikoff, 2014), Fig. 1]. Most importantly, since epigenetic marks constitute dynamic and potentially reversible modifications, they represent outstanding candidates for developing fast and sensible environmental biomonitoring programs in diverse ecosystems (Dolinoy and Jirtle, 2008; Huang et al., 2012).

2. Marine invertebrate models in environmental epigenetics

Oceans bear the brunt of climate change, as evidenced by growing pollution and acidification levels, sea level increase, and changes in temperature and currents. Altogether, these factors impact the health of marine species, ecosystems, and coastal communities, making oceans one the most important targets for environmental studies (Reid et al., 2009). Among these, pollution has critical consequences due to its inherent deleterious genotoxic effects on marine life, triggering adaptive responses that often involve extensive genetic reprogramming in order to preserve genome integrity (Liu et al., 2010). Therefore, the study of the cause-effect links between pollutants (especially those encompassing genotoxic potential, e.g., Polycyclic Aromatic Hydrocarbons discharged during oil spills and marine biotoxins produced during

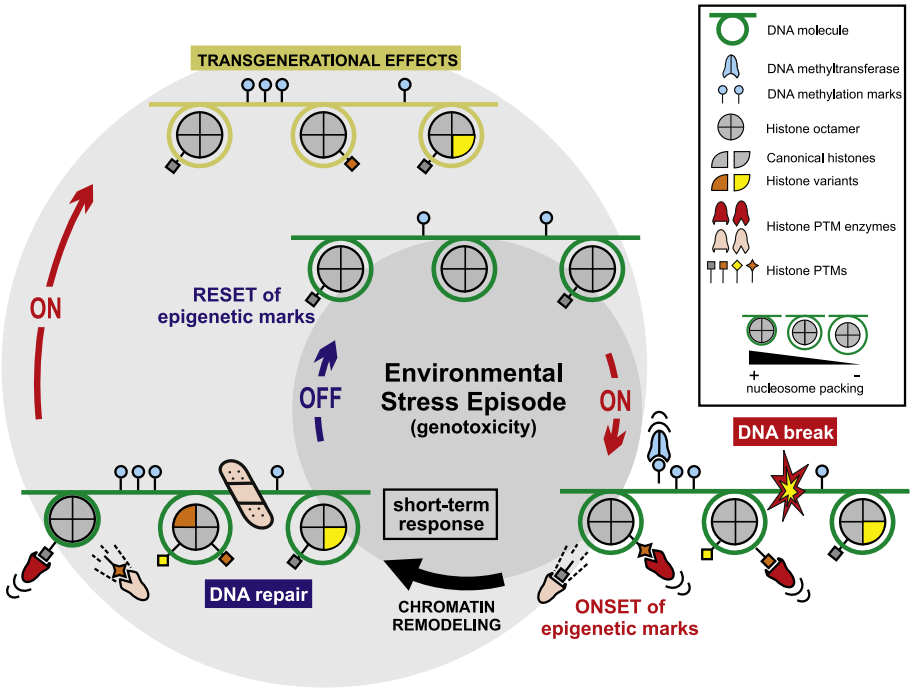

Fig. 1. Epigenetic modifications transmit external signals to DNA. Environmental changes require swift genetic responses in the cell, notably in those cases where genotoxic stress is involved (see example in the picture). Within the cell nucleus, stress episodes (e.g., DNA breaks) will be met by the onset of epigenetic modifications triggering the remodeling of the chromatin fiber (condensation/decondensation) and thus modulating the access to specific genes involved in the response to DNA damage. These modifications include DNA methylation (usually associated with gene silencing), replacement of canonical histones by histone variants with dedicated functions in the nucleosomes, and enzymes adding and removing post-translational modifications at specific residues in histone tails (see legend on the right margin of the figure for details). Overall, different marks will result in specialized epigenetic states across the genome, facilitating DNA repair. Once the stress episode is over most of these marks will be reset, reverting the genome-wide structure of the chromatin fiber to its basal state before the damage. Nonetheless, many of these epigenetic marks will transcend throughout generations in those cases where the environmental stress persists, securing a continuous response to genotoxicity in the cell and establishing the basis for organismal long-term adaptations.

Harmful Algal Blooms) and the biological responses displayed by marine organisms encompasses dual benefits: first, it sheds light into the epigenetic mechanisms underlying environmental adaptive responses; and second, it provides a promising venue for developing fast and sensible pollution biomonitoring programs in the oceans (González-Romero et al., 2012a). Marine invertebrates, the largest group of macroscopic organisms in the oceans (Ruppert et al., 2004), are commonly used as model systems in such studies because of their ubiquitous distribution, easy accessibility and diverse lifestyles including sessile filter-feeding organisms (Gosling, 2003). Among them, bivalve molluscs stand out as model sentinel organisms for the study of pollution, particularly in coastal areas where they constitute valuable commercial resources for the aquaculture industry (Collin et al., 2010; Campos et al., 2012; Fernandez-Tajes et al., 2012; Luchmann et al., 2012; Milan et al., 2013; Suarez-Ulloa et al., 2013a).

The feasibility of environmental epigenetic studies in marine organisms is currently supported by the availability of high-throughput data and computational resources, including the ongoing characterization of several genomes in cnidarians, ctenophores, molluscs, echinoderms and other chordates (Sodergren et al., 2006; Putnam et al., 2007, 2008; Zhang et al., 2012; Moroz et al., 2014). More specifically, DNA methylation has been recently studied using methylation-specific restriction enzymes (del Gaudio et al., 1997; Diaz-Freije et al., 2014; Sun et al., 2014; Zhao et al., 2014) and genome-wide bisulfite sequencing (Zemach et al., 2010; Gavery and Roberts, 2013; Huang et al., 2014). Similarly, structural, functional and evolutionary aspects of chromatin are also being currently studied in this group of organisms (Eirín-López et al., 2002, 2004, 2006, 2009; González-Romero et al., 2009, 2012b) unveiling the presence of specialized histone variants including H2A.X, H2A.Z and H3.3 (Arenas-Mena et al., 2007; Schulmeister et al., 2007; González-Romero et al., 2012b), as well as possibly macroH2A and other variants (work in progress). Most importantly, biochemical and transcriptomic analyses suggest that histone variants from marine invertebrates are able to specialize chromatin (González-Romero et al., 2012b) and that several chromatin-associated genes are differentially regulated in response to environmental signals in these organisms (Suarez-Ulloa et al., 2013a). Overall, these results support the role of marine invertebrates as model systems for environmental epigenetic studies.

3. Background on environmental epigenetic studies

Environmental epigenetics represents an emerging field and as such, research efforts are still unevenly distributed across different groups of organisms, environmental factors and epigenetic mechanisms. Epigenetic biomarkers are now within reach in vertebrate model organisms [e.g., human, mouse, zebrafish (Hou et al., 2011; Ho et al., 2012; Williams et al., 2014)], where high-throughput methods have been applied to study the epigenetic basis underlying responses to pesticides (Song et al., 2010), PAHs (Marwick et al., 2004; Kikuchi et al., 2006; Alegria-Torres et al., 2013; Fang et al., 2013), and heavy metals (Santoyo et al., 2011; Gadhia et al., 2012; Basu et al., 2013). Furthermore, the predictive power of gene expression profiles using arrays has been demonstrated *in vitro* and *in vivo*, predicting not only toxicity but also discriminating among toxicants according to their mechanisms of action [(Burczynski et al., 2000; Waring et al., 2001; Hong et al., 2003; Elferink et al., 2008; Inadera et al., 2008), Fig. 2]. Nowadays, this goal is routinely approached using omic techniques that include RNA-Seq (transcriptomics) and high-throughput Mass Spectrometry (MS, proteomics). Accordingly, transcriptomics and proteomics have been applied to detect exposure to environmental pollutants in marine invertebrates including mussels and oysters (Suarez-Ulloa et al., 2013b), as well as in several other marine organisms (Schirmer et al., 2010; Slattery et al., 2012). It seems, based on the growing ability to generate and analyze high-throughput data in a broader range of organisms, that the future development of epigenetic biomarkers will walk hand in hand with these technologies (Vandegehuchte and Janssen, 2014).

Environmental epigenetic analyses have also been implemented in other aquatic (freshwater) invertebrates. Among these organisms, the water flea *Daphnia* is probably the best characterized, constituting an emerging model for pollution biomonitoring in freshwater environments (Harris et al., 2012). Indeed, the exposure of *Daphnia* to chemical pollutants has been shown to cause epigenetic modifications inherited throughout different flea generations (Vandegehuchte et al., 2009, 2010). Nevertheless, the amount of epigenetic knowledge in invertebrates is still pale in comparison with vertebrates. Fortunately, several recent reports have started to address different aspects related to the epigenetic

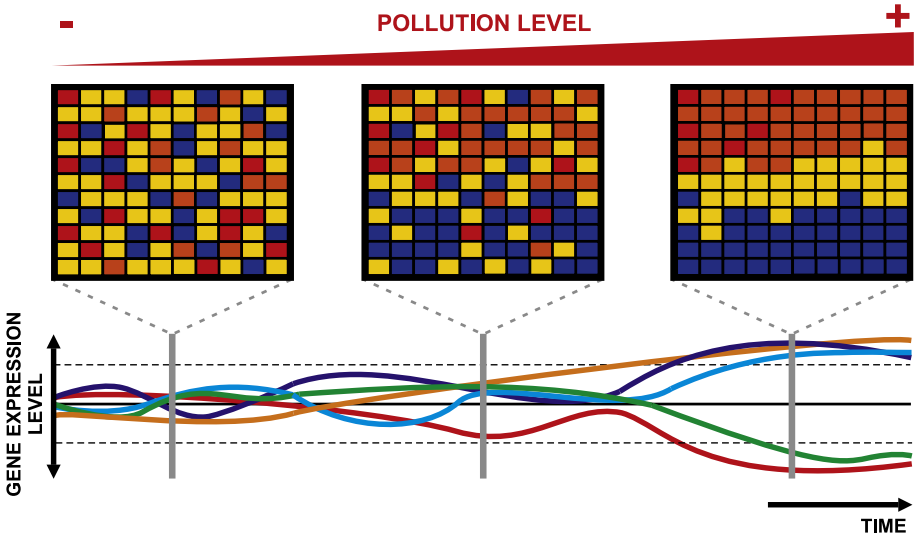

Fig. 2. Pollution biomonitoring tools. The systematic monitoring of marine pollution using microarray technologies allows to organize data into time series. Such analytical approach is useful to investigate the expression levels of individual and/or groups of relevant genes. In combination with the development of *ad hoc* software, the application of microarrays will help identify genes undergoing diagnostic changes in expression levels in response to specific pollutants, constituting candidate biomarkers.

Table 1
Summary of environmental epigenetic studies using marine invertebrates as model organisms.

Species	Group/COMMON name	Epigenetic mechanism	Major finding	Reference
Bugula neritina	Bryozoan	Transgenerational adaptive mechanisms	Context-dependent transgenerational effects in response to copper exposure	Marshall (2008)
Hydroides diramphus	Polychaete	Transgenerational adaptive mechanisms	Adaptive gamete plasticity in response to salinity stress	Jensen et al. (2014)
Spiophanes tcherniai	Polychaete	DNA methylation	Genome-wide DNA methylation in response to thermal stress	Marsh and Pasqualone (2014)
Crassostrea gigas	Mollusc (Pacific oyster)	DNA methylation	Intragenic DNA methylation linked to adaptation	Gavery and Roberts (2010, 2013)
Chlamys farreri	Mollusc (Zhikong scallop)	DNA methylation	Tissue-specific gene expression regulated by DNA methylation	Sun et al. (2014)
Mytilus galloprovincialis	Mollusc (Mediterranean mussel)	Histone variants and PTMs	Characterization of H2A.X and H2A.Z histone variants	González-Romero et al. (2012b)
Crassostrea gigas	Mollusc (Pacific oyster)	Histone variants and PTMs	Modification of H3 methylation in response to thermal stress	Fellous et al. (2015)
Pseudocalanus acuspes	Copepod	Transgenerational adaptive mechanisms	Transgenerational effect of ocean acidification in two consecutive generations	Thor and Dupont (2015)

mechanisms involved in environmental responses in marine invertebrates. The most relevant are described below and summarized in Table 1.

3.1. DNA methylation

So far, the bulk of the epigenetic studies developed in marine invertebrates has been essentially focused on DNA methylation (the addition of methyl groups to cytosine nucleotides), with studies on histone variants and their modifications still on the background (del Gaudio et al., 1997; Arenas-Mena et al., 2007; Schulmeister et al., 2007; Gavery and Roberts, 2010; Zemach et al., 2010; González-Romero et al., 2012b; Diaz-Freije et al., 2014; Huang et al., 2014; Zhao et al., 2014). DNA methylation at CpG islands of gene promoters constitutes a mark characteristic from vertebrate organisms (Deaton and Bird, 2011; Su et al., 2011). On the contrary, invertebrates often display DNA methylation predominantly within gene bodies, associated with gene expression regulation and alternative splicing (Su et al., 2011; Gavery and Roberts, 2013). Nonetheless, studies of DNA methylation in the oyster *Crassostrea gigas* suggest that gene promoter methylation can also be relevant for evolution and adaptation (Riviere, 2014).

Several studies account for the potential role of DNA methylation during environmental adaptation in marine invertebrates. For instance, it has been reported that the increase in water temperature correlates with a net increase in genome-wide methylation in the marine polychaete *Spiophanes tcherniai* (Marsh and Pasqualone, 2014). Nonetheless, the cause-effect correlation between temperature and DNA methylation is still uncertain, as detailed analyses are hampered by the lack of genomic information in this organism. On the contrary, genome-wide DNA methylation analyses are possible and already available in the Pacific oyster *C. gigas* and the Zhikong scallop *Chlamys farreri* (Gavery and Roberts, 2013; Sun et al., 2014), establishing links between DNA hypomethylation and transcription of genes potentially linked to phenotypic plasticity and adaptation (Gavery and Roberts, 2010). These studies represent a leap forward from previous DNA methylation studies in bivalves (Wang et al., 2008; Petrovic et al., 2009), providing a very powerful platform to study its regulatory role during environmental responses and adaptation.

While the characterization of DNA methylation in marine invertebrates seem to be on its way, several relevant questions still remain unanswered. For instance, what percentage of DNA methylation persists across different marine invertebrates?, where is it localized in the genome?. Similarly, while DNA methylation has been extensively described in marine invertebrates, the specific links between specific methylation patterns (Lirman and Cropper, 2003) and particular environmental factors still remains obscure. The answers for these and many other questions will require further studies, especially those combining experimental and *in silico* analyses able to characterize DNA methylation levels, specific patterns and their variation across a broader range of invertebrates from diverse environments.

3.2. Histone variants and post-translational modifications

The presence of specialized histone variants in marine invertebrates was not fully known until the presence of functional H2A.X and H2A.Z was unequivocally demonstrated in molluscs (González-Romero et al., 2012b). Such discovery has fueled further analyses (many still on the make) suggesting that the diversity of variants in these group might be broader than previously thought, including H3.3 as well as other variants traditionally reserved for vertebrates such as macroH2A (work in progress). Concomitantly, biomonitoring studies using bivalve molluscs have evidenced conspicuous modifications in the expression of some of these variants in response to genotoxic marine biotoxins. Interestingly, such changes were accompanied by modifications in histone post-translational modifications such as H3 phosphorylation (work in progress). Following in this direction, a recent report has also revealed changes in histone H3 methylation and in the expression of the Jumonji histone demethylase in embrionic and early larval stages of the Pacific oyster *C. gigas* in response to changes in external temperature (Fellous et al., 2015).

3.3. Transgenerational adaptive mechanisms

In addition to tracing relationships between environment and epigenetic modifications, one of the most interesting and challenging goals of environmental epigenetics is to elucidate how epigenetic modifications are transmitted across generations and their role in the acquisition of long term adaptations. Such objective is often pursued by studying phenotypic alterations in the offspring of individuals exposed to challenging environmental conditions. Accordingly, the effects of pre-reproductive salinity stress were evaluated in the marine tubeworm *Hydroides diramphus*, revealing transgenerational alterations in gamete phenotype and offspring development (Jensen et al., 2014). Similarly, transgenerational modifications have also been described in the bryozoan *Bugula neritina* after exposure to copper (Marshall, 2008). Nonetheless, it has been argued that phenotypic modifications must persist for

at least two consecutive generations in order to be considered *bona fide* transgenerational effects (Feil and Fraga, 2011). That concern was addressed by a study investigating the response of the copepod *Pseudocalanus acuspes* to ocean acidification, finding evidence supporting the transmission of physiological responses to high CO_2 pressures through generations F1 and F2 (Thor and Dupont, 2015).

While these results are promising, further efforts are still necessary in order to unequivocally elucidate the links between environmental factors, specific epigenetic marks and the subsequent phenotypic modifications leading to long term adaptations in marine invertebrates. Such work is challenging, specially at the time of following the scent of different epigenetic marks across consecutive generations and, most importantly, at the time of doing that outside the lab (i.e., in the field). Different studies have dealt with this problem in different ways. For instance, the study of transgenerational modifications in bryozoans (Marshall, 2008) resorted to methods controlling spawning and gamete/larvae dispersion. Similarly, the control of gamete dispersion, together with the analysis of epigenetic modifications in the germline, constitute the methods most commonly used in plants (Verhoeven et al., 2010; Crevillen et al., 2014; Herrera et al., 2014). On the contrary, DNA paternity tests and isotopic labeling have been predominantly used in studies focused on mobile organisms and sessile species with high-dispersive seeds (Cuif et al., 2014; Evans et al., 2014). Overall, the combination of laboratory work with field experiments holds the key to ascertain the true biomonitoring potential of different epigenetic marks and their applicability to different environmental contexts. Although such potential will ultimately depend on the cost and expertise required for introducing epigenetics as a biomonitoring tool in the field, the low sequencing costs and the increased automation of data analyses strongly support this option.

4. Future perspectives

4.1. Experimental challenges

The epigenetic knowledge currently available in marine invertebrates constitutes an exciting springboard for prospective environmental studies. However, in order to establish correlations between environmental factors and specific epigenetic states, it is still necessary to gain information about the genomic position of different epigenetic marks, in other words, the characterization of the epigenome. The recent characterization of genome-wide patterns of DNA methylation (methylome) at single base-pair resolution in the Pacific oyster (Gavery and Roberts, 2013) has brought significant progress in that direction. Combined with the study of locus-specific DNA methylation using methylation-specific PCR (MSP) assays (Candiloro et al., 2011; Ku et al., 2011) coupled to real-time monitoring of PCR amplifications (Eads et al., 2000), these techniques can provide validated quantitative information useful to study environmental epigenetic responses. Complementarily, the development of antibodies specifically raised against histone variants from marine invertebrates [e.g., mussel H2A.Z (González-Romero et al., 2012b)] will facilitate the identification of genes enriched in specific chromatin fractions, notably through chromatin immunoprecipitation [ChIP (O'Geen et al., 2011)] and high-throughput DNA sequencing (ChIP-Seq) techniques. These approaches represent very powerful tools for targeting proteins and PTMs specifically involved in responses to environmental stress (Li et al., 2005).

The characterization of the epigenetic role of histone variants and their PTMs represents a challenging task requiring detailed structural and functional knowledge of chromatin in marine invertebrates. These analyses are not trivial, as they involve working with two complementary epigenetic regulatory layers: First, the characterization of structural transitions resulting from the recruitment of histone variants into chromatin requires nucleosome reconstitution experiments. In this sense, previous reports have demonstrated that promoter regions of environmentally responsive genes could represent suitable templates for such purposes (González-Romero et al., 2012b). Combined with electrophoretic mobility analyses and quantitative biophysical approaches (e.g., circular dichroism and analytical ultracentrifugation), this strategy has proven to be the most powerful in ascertaining changes in nucleosome structure, helping establishing links between specific epigenetic marks and the subsequent modifications in chromatin structure and gene expression (Ausio, 2000; Thambirajah et al., 2005). Second, the nature and position of PTMs on canonical histones and histone variants must be evaluated, specially those modifications known to be involved in the maintenance of genomic integrity during genotoxic episodes [e.g., serine phosphorylation in H2A.X and H3.3 (Li et al., 2005)].

4.2. Integration and interpretation of epigenetic and epigenomic data

Most traditional toxicogenomic studies aim to find biomarkers within a single group of biomolecules [e.g., transcripts, proteins, ncRNA (Hou et al., 2011; Gadhia et al., 2012)], or chemical marks [predominantly DNA methylation (Kikuchi et al., 2006; Santoyo et al., 2011; Herbstman et al., 2012; Alegria-Torres et al., 2013; Basu et al., 2013)]. Such strategy bears obvious limitations at the time of studying different epigenetic states, as these are often dictated by the combination of different types of mechanisms. Consequently, the development of powerful epigenetic biomarkers requires the simultaneous characterization of different types of marks and the subsequent integration and interpretation of the resulting data. It seems therefore that the progress of environmental epigenetics will rely heavily on the generation and integration of the different types of omic data constituting the epigenome. However, while the holistic study of the epigenome constitutes a powerful tool, it also poses new challenges, especially at the time of organizing and analyzing the immense amount of high-throughput information generated during environmental studies.

A possible strategy to tackle this problem is shown in Fig. 3. Accordingly, high-throughput omic data [methylome, transcriptome, and proteome (Robinson et al., 2009; Anders and Huber, 2010; Hardcastle and Kelly, 2010; Tarazona et al., 2011)] must be compared between organisms exposed and non-exposed to specific environmental conditions in order to identify differential gene expression patterns (Manfrin et al., 2010; Banni et al., 2011; Aguiar-Pulido et al., 2013b). The success of this approach is ultimately subject to the implementation of appropriate data mining techniques (Bock and Lengauer, 2008; Aguiar-Pulido et al., 2013a) and the organization of this information into databases, facilitating the integrative study of gene interactions and regulatory mechanisms involved in the response to specific pollutants. The Human Epigenome Consortium is at the forefront of this research, developing bioinformatic frameworks for data integration, standardization and producing reference epigenomic maps representing specific cellular conditions related to health or disease (Bae, 2013). Integrative analyses have also been recently expanded to environmental studies, as illustrated by the Comparative Toxicogenomics Database, addressing cause-effect relationships between abiotic factors and human health (Mattingly et al., 2003; Davis et al., 2013). Although heavily oriented to humans, these tools are paving the road to expand these analyses to a broader range of organisms, specially those encompassing relevance for pollution biomonitoring in the marine environment. As an example, the biomarker potential of chromatin-related genes, differentially expressed in response to marine biotoxins, is currently under investigation in bivalve molluscs (Suarez-Ulloa et al., 2013a).

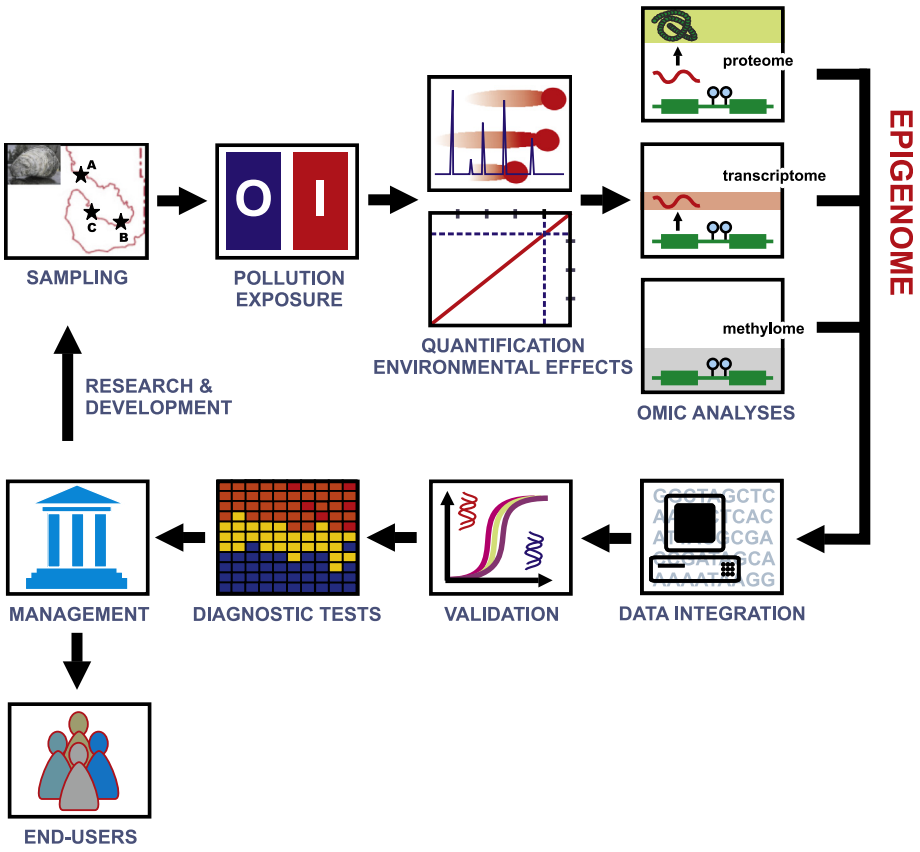

Fig. 3. The integrative analysis of omic data is fundamental for the genome-wide characterization of epigenetic marks. The generation of high-throughput sequence data and its comparison between control (O) and exposed (I) individuals provides information about the genetic factors participating in the response to specific pollutants. To this end it is necessary to set up optimal experimental conditions able to efficiently simulate pollution episodes in the laboratory (e.g., HABs, oil spills) triggering specific transcriptional responses in model organisms. This task requires homogeneous exposure of control and treated groups to pollutants of interest (Florez-Barros et al., 2011; Suarez-Ulloa et al., 2013a), followed by their quantification at different intervals using chemical [e.g., direct quantification (McNabb et al., 2012)] and biological [quantification of resulting DNA damage (Fernandez-Tajes et al., 2011)] methodologies. The epigenetically relevant omes (methylome, transcriptome and proteome) can be then studied, producing qualitative and quantitative data for further processing and analysis. At this point, the heterogeneity of datasets obtained from different omes will require of specialized bioinformatic techniques for their integration. After validation, the obtained patterns can be used to create a computerized models to interpret routinary analyses for an automated monitoring of pollution levels. This tool has dual benefits, on one hand it has positive impacts on end-users and stakeholders in different industries (e.g., aquaculture and fisheries); on the other, it provides a framework for developing further research geared toward the characterization of new biomarkers in additional species/environments.

5. Conclusions

The epigenetic characterization of ecologically relevant organisms is paving the road toward the characterization of cause-effect relationships between environmental factors and epigenetic mechanisms involved in immediate responses and long term adaptations. This approach, in combination with high-throughput analytical methods and the bioinformatic integration of different types of omic data, lays the foundations for developing a new generation of biomarkers of marine pollution based on dynamic epigenetic modifications. While this goal is already a reality in several vertebrate model organisms, its development in marine invertebrates still awaits further studies facilitating genome wide analyses of epigenetic marks, including DNA methylation and modifications in chromatin structure and dynamics. Most importantly, research efforts investigating the transmission of these marks across generations will be critical to unravel the role of epigenetics in adaptation.

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