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VISIONS AND PERSPECTIVES

Concepts and tools for exploiting sessile bio-filters as early warning elements: introductory applications for marine ecosystem preservation

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Abstract

Current evidence suggests that integrating diverse warning systems at different biological levels may not only increase the probability of detecting threats but also mitigate their impact. Here, we propose the use of both molecular and morphological descriptors at different biological levels in sessile bivalves (a suitable biological model in monitoring programs) to collect information on the ecosystem health of coastal marine habitats. In this context, studies may be implemented on biomarkers to exploit some population features, with the aim to propose an actual monitoring program that predictively would provide possible scenarios on the species fitness and ecosystem changes. Thus, the use of quality biotic elements may provide an objective environmental monitoring method and facilitate the development of sanitary, economic, and social strategies related to sustainable exploitation.

Key Words: cellular and morphological biomarkers; environmental status; long- and short-term disturbances; *Mytilus galloprovincialis*; monitoring

Introduction

A number of international meetings have recently taken place with the goals of reducing human impacts on ecosystems and living resources and ameliorating the effects of natural resource exploitation. Such meetings are being held at an increasing frequency largely in response to the United Nations Conference on Environment and Development in Rio de Janeiro, Brazil (UNCED, 1992). This conference provided the impetus to stimulate politicians, scientists, and socio-cultural and socio-economic managers toward developing HORIZON 2020, a recently created European Program for Research and Innovation proposed by the European Parliament (2011).

Several attempts have been made to prevent, ameliorate or remedy the effects caused by human impact. In this context, the use of living systems approach (across various levels of biological organization) may be as important as the development of environmental monitoring tools (Fasulo *et al*., 2015). Indeed, biological models can act as descriptors of environmental health and serve as ‘early warning systems’ (EWSs). As an example, in marine habitats, many benthic taxa have been used frequently as sentinel organisms in monitoring programs to evaluate the impact of anthropogenic disturbances. The latter is essentially due to their life-history characteristics and to their relatively rapid response to anthropogenic and natural stress descriptors (Roméo and Gnassia-Barelli, 1997; Cheggour *et al*., 2001; Griscom *et al*., 2002; Lecoeur *et al*., 2004; Baudrimont *et al*., 2005; Machreki-Ajmi and Hamza-Chaffai, 2008; Smaoui-Damak *et al*., 2009; Serafim and Bebiano, 2010; Ramos-Gomez *et al*., 2011; Wang *et al*., 2012).

Two different types of EWSs, biomarkers and bioindicators, can be used to detect effects at different biological levels. The redefinition of both were first proposed by Van Gestel and Van Brummelen (1996) and then modified (and internationally accepted) by Peakall and Walker (1996). Specifically, a biomarker is described as a set of responses to chemical exposure at the individual/sub-individual (molecular, cellular, histological, physiological, anatomical, morphological, and behavioral) level. Instead, bioindicators can be considered as responses to changes of environmental conditions at the levels of population, species, community, ecosystem, and landscape (the latter in Adams *et al*., 2001). Then, biomarkers respond rapidly to stressors but often

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provide less information on their effects at ecosystem level. Conversely, while bioindicators provide direct information on the habitat status in terms of environmental health, they often show a long response time to environmental changes. This is why McCarty *et al.* (2002) emphasized the usefulness of bioindicators to address environmental risk issues and, at the same time, they stated that the current practice tends to focus on the use of biomarkers. Practically, biomarkers better reflect short-term effects (STEs) to direct exposure, while bioindicators are more appropriate to highlight long-term effects (LTEs). Thus, STEs and LTEs may operate at different levels of biological organization. Nevertheless, changes at the molecular and cellular levels may drive consequences at population, community or ecosystem levels, based on bottom-up effects (Lagadic *et al*., 2000; Hagger *et al*., 2008). However, some difficulties can emerge in developing investigations on stressor-specific, and quantitative dose-response functions to assess environmental risks (see Bartell, 2006). Therefore, an integrated approach to evaluating the ecosystem quality is becoming a must in biomonitoring, thereby helping to detect pollution-induced effects at several levels of the biological organization (Allen and Moore, 2004). Thus, the use of biomarkers and bioindicators in combination is needed for effective ecosystem monitoring and the prevention of adverse biological effects.

Environmental pollutants produced by the settlements of approximately three billion people (with their associated agricultural and industrial activities and domestic effluents) are continuously being discharged into the marine ecosystem (Moore *et al*., 2004); as such, it is easy to understand why aquatic habitats are considered amongst the most imperiled (Olson *et al*., 2002). Exacerbating this situation, coastal systems are preferred by many species, some with high economic value, for reproduction, nursery, and maturation. Because the sediments of coastal ecosystems act as a sink for a variety of contaminants, benthic fauna in particular are regularly exposed to contamination stressors (Langston *et al*., 2010). Although the disposal of polluting substances and systems via wastewater management mitigate human impact, it has become necessary to better understand and thereby prevent harmful environmental exposures. The Water Framework Directive (WFD) of the Council of the European Parliament (2000/60/EC) was a fundamental step in promoting bio-monitoring and aquatic habitat restoration.

Here, we evaluate the use of biomarkers at different biological levels and apply morphological and molecular markers to investigate the ultimate repercussions of using this approach to monitor population characteristics such as growth, structure and dynamics (the latter being exploited for describing population predictive models). Specifically, we propose an integrated approach exploiting sessile bivalves as biological EWSs to contribute in monitoring the coastal marine habitat health.

A suitable biological model: the mussel Mytilus galloprovincialis

The use of bio-monitoring requires the following actions: 1) choice of bio-model, 2) assessment of type and time of response to a specific stressor, 3) selection of protocols and tools (such as biotic indices and/or molecular threshold), and 4) management of environmental preservation and mitigation. Choosing an adequate biological model is important. No model is universal because different organisms show diverse degrees of habitat adaptation. Moreover, the choice of a model depends on the habitat being investigated, as well as on the operator's expertise in both an environment and the organisms living therein.

The structural and trophic complexities of marine habitats constrain the type of biological models can be used as potential EWSs. Due to their life-history characteristics and relatively rapid responses to anthropogenic and natural stresses, members of the benthic community, especially bivalves (Griscom *et al*., 2002; Wang *et al*., 2012), have frequently been used as sentinel organisms in monitoring programs. The mussel *M. galloprovincialis*, a common sessile bivalve that is easy to identify and collect, is among the most exploitable biofilters around the world and has considerable economic importance in terms of both aquaculture (its value as a harvested wild resource), and an EWS in coastal monitoring projects (*e.g.,* Hagger *et al*., 2008; Stankovic *et al*., 2011). Indeed, *M. galloprovincialis* is a widespread and long utilized biological model in scientific projects funded at national and international level, promoted by different organizations (see protocols of United Nations Environment Programme - Mediterranean Action Plan), and actually used within the project 'Systems Biology' (funded by Ministry of Education, University and Research, MIUR-PRIN, Italy), of which we are a functional operative unit (Fasulo *et al*., 2015).

Acting as a sentinel organism, this bivalve species can help detect marine pollution because of its filter-feeding activity, capacity to bioaccumulate chemicals (Cheggour *et al*., 2001; Metian *et al*., 2009), sedentary lifestyle, widespread distribution, and abundance. Thereby, bivalves can provide a time-integrated indicator of environmental contamination (Figueira *et al*., 2011).

Specimens collected in the field and sacrificed for molecular and morphological assays should therefore provide an immediate response to acute and intense stress (short-term effects, STE) and an indication of chronic exposure to pollutants (long-term effects, LTE).

Short-term effects

Cellular markers

Evaluating molecular responses to pollutants is an emerging method of assessing the impact of stress on the environment (Pampanin *et al*., 2005; Regoli and Giuliani, 2014; Benedetti *et al*., 2015). Exposure to xenobiotics is commonly associated with the production of radical oxygen and nitrogen species (ROS/RNS), including superoxide radicals, hydrogen peroxide, nitric oxide, and peroxynitrite, which can impair cell signaling, alter gene regulation and cause cellular damage via protein oxidation, enzyme inhibition, lipid peroxidation and DNA damage. It is notable that antioxidant enzymes, including catalase and superoxide dismutase (SOD), provide primary defenses against oxidative damage, thereby increasing the tolerance of organisms to polluted environments and their persistence therein. However, chronic oxidative stress occurs when cellular homeostasis is altered because of persistent accumulation of contaminants and subsequent excessive production of ROS/RNS and/or impairment of cellular antioxidant defenses (Valavanidis *et al*., 2006). Conversely, recent data indicate that transcriptional and catalytic responses to environmental stressors do not necessarily correspond to functional changes (Giuliani *et al*., 2013). An increasing number of studies have focused on the assessment of post-translational modifications (PTMs) of proteins as a signal of toxicity. In particular, ROS/RNS can cause specific reversible and irreversible oxidative modifications to sensitive proteins that may change their activities and functions. Under conditions of oxidative/nitrosative stress, thiols in cysteine residues within proteins are among the most susceptible oxidant-sensitive targets. These thiols undergo various reversible and irreversible redox alterations in response to ROS and/or RNS production (Fig. 1). Reversible oxidation, such as that occurring during *S*-nitrosylation, *S*-glutathionylation and the formation of inter- or intra-disulfide bonds, can play regulatory roles both in sub-stress scenarios and during organismal responses to acute oxidative stress (Ascenzi *et al*., 2000; Musci *et al*., 2006; Dalle-Donne *et al*., 2007; Casadei *et al*., 2008). Alternatively, chronic oxidative stress caused by specific pollutants can result in irreversible oxidation (*e.g*., protein carbonylation), leading to protein aggregation, inactivation and degradation.

Bivalves can be exposed to relatively high levels of pro-oxidants as a consequence of their filter-feeding activities. Common pro-oxidant environmental pollutants, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), heavy metals and organochlorines, are bioconcentrated within bivalves and lead to oxidative stress (McDonagh *et al*., 2006). Previously, a 2DE proteomic approach was combined with immunoblotting to investigate carbonylation in individual tissues in response to a variety of stressors and pollutants known to induce oxidative stress in both mussels and clams (McDonagh *et al*., 2005; McDonagh and Sheehan, 2006). Furthermore, increased *S*-glutathionylation of proteins was observed in gill tissues of *Mytilus edulis* in response to H_2O_2 and menadione treatment (McDonagh *et al*., 2005). Numerous protein species (*e.g.,* actin, protein disulfide isomerase (PDI), and other chaperones) in *Mytilus* form intramolecular disulfide bonds following exposure to menadione-induced ROS (McDonagh and Sheehan, 2007, McDonagh and Sheehan, 2008).

Thus, in the current proposal, we aim to identify protein species (*e.g.,* actin, HSP70, carbonic anhydrase, catalase, SOD, PDI, glutathione-S-transferase, and troponin) that can serve as molecular biomarkers due to their tendencies to undergo PTM induced by ROS/RNS. Some of these proteins may represent specific sub-proteomes for diverse xenobiotics.

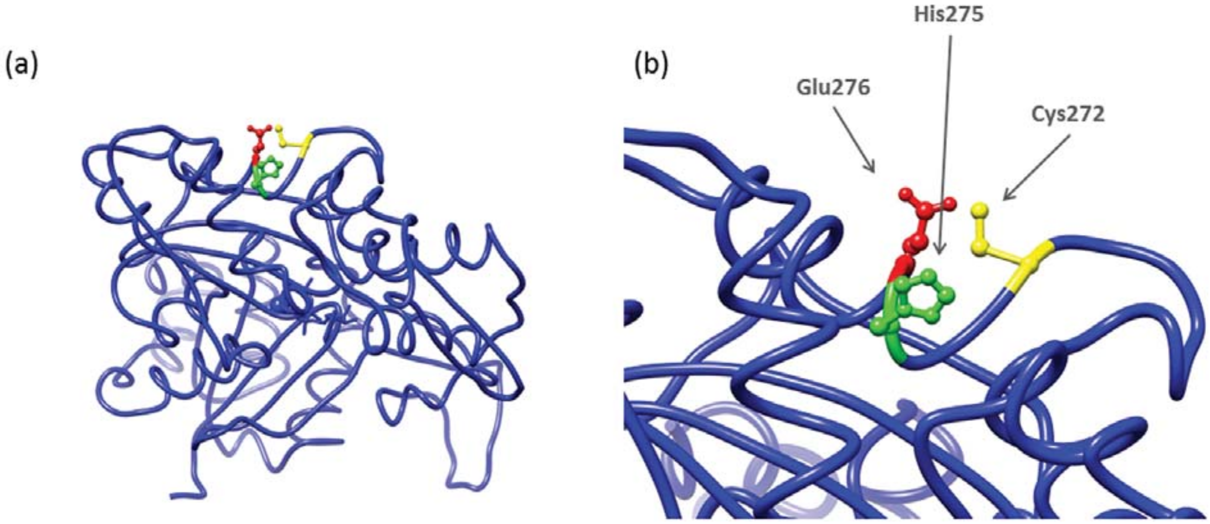

Fig. 1 (a) Ribbon diagram of β-actin (PDB ID: 2BTF); (b) Cys272, His275 and Glu276 residues are shown in ball-and-stick representation. The thiol in Cys272 is an oxidant-sensitive target (*i.e., S*-nitrosylation and *S*-glutathionylation). The image was generated using the program CHIMERA (Pettersen *et al*., 2004).

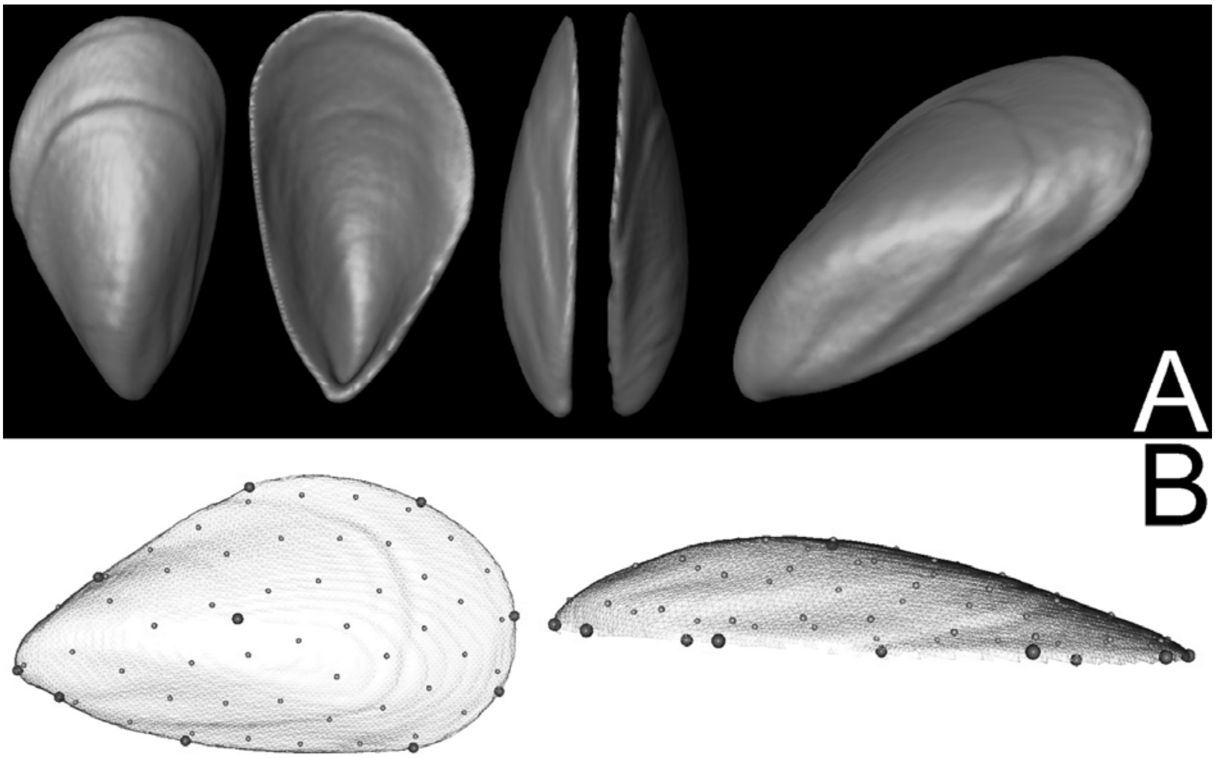

Fig. 2 A) Three-dimensional surface scans of a representative mussel valve. B) The positions of 10 landmarks (larger, darker circles) and 50 semi-landmarks (smaller, lighter circles) are shown.

Long-term effects

Morphological markers

Shell shape is a morphological indicator of stress in bivalves. In addition to markers recorded by eye or by traditional measurements, we focused on novel morphometric biomarkers detectable in bilateral organisms. Slight asymmetries in Bilateria may reflect an organism's ability to overcome the effects of stress. Thus, organisms deviating from perfect symmetry by showing either fluctuating asymmetry (*i.e.,* non-directional deviation from perfect symmetry) or directional asymmetry (*i.e.*, more growth on one side than the other) (Graham *et al*., 2010) may be associated with some type of developmental noise. Morphological asymmetries may therefore be useful as a general biological stress indicator and represent a suitable marker for LTE (Arambourou *et al*., 2012).

To investigate morphological markers, landmark-based geometric morphometric methods can be used to quantify shell shape deformations. In particular, the spatial coordinates of three-dimensional homologous locations (*i.e.,* landmarks) and points that depend on them (*i.e.*, semi-landmarks) can be easily exploited after removing non-shape effects [for more details, see (Adams *et al*., 2004)].

In this context, captured images are analyzed based on spatial relationships between Cartesian coordinates of landmarks and semi-landmarks. Once a set of points (landmarks + semi-landmarks) is fixed on an image using specific software, the x-y-z Cartesian coordinates of each point are registered to obtain the distribution of the points on the studied surface (*i.e.,* their configuration). The landmarks and semi-landmarks used to quantify shell shape and surface texture in the present proposal were chosen because strict homology in the investigated structures could not be determined (Fig. 2); these landmarks have been previously utilized (Serb *et al*., 2011) to capture general shell surface shape. An important advantage of this approach is that shape information from landmarks, points along curves and points on anatomical surfaces can be included in the same analysis.

Finally, all of the configurations of each specimen may be aligned using a generalized Procrustes superimposition, a procedure that removes information on location orientation and rotation, and standardizes each specimen to a unit centroid size (CS - *i.e*., the square root of the summed squared Euclidean distance from each landmark to the specimen centroid, which provides an estimate of the size of the studied structure). During this procedure, semi-landmarks were permitted to slide along their tangent directions to minimize the Procrustes distance between specimens. From the aligned configurations, a set of Procrustes shape coordinates was obtained and used as shape variables in subsequent multivariate statistical analyses (Mitteroecker and Bookstein, 2008). The Euclidean (Procrustes) distances amongst the individuals in the morpho-space may be calculated to compare findings within and among groups. Warp grids representing shape deformations can be associated with diagrams resulting from multivariate analyses of ordination to visualize morphological variation patterns and facilitate biological interpretation.

Population structure and dynamics

Studying valve structure also provides an opportunity to better understand population structure and dynamics. Because this biological feature is considered an essential aspect of fishery management, assessments of population structure and dynamics can be used as tools to monitor the effects of external stressors on valve structure. To study these structures, we first decompose a population into cohorts defined by age classes. Age can be evaluated by using external tags during recapture or by assessment of body size (i.e., shell size). Several methods of length frequency analysis using modal progression techniques can be used to perform aging analyses, and aging mussels is a relatively easy undertaking due to the frequent use of *M. galloprovincialis* in aquaculture. Therefore, is straightforward to create growth rate simulations of reared specimens (see Fig. 3).

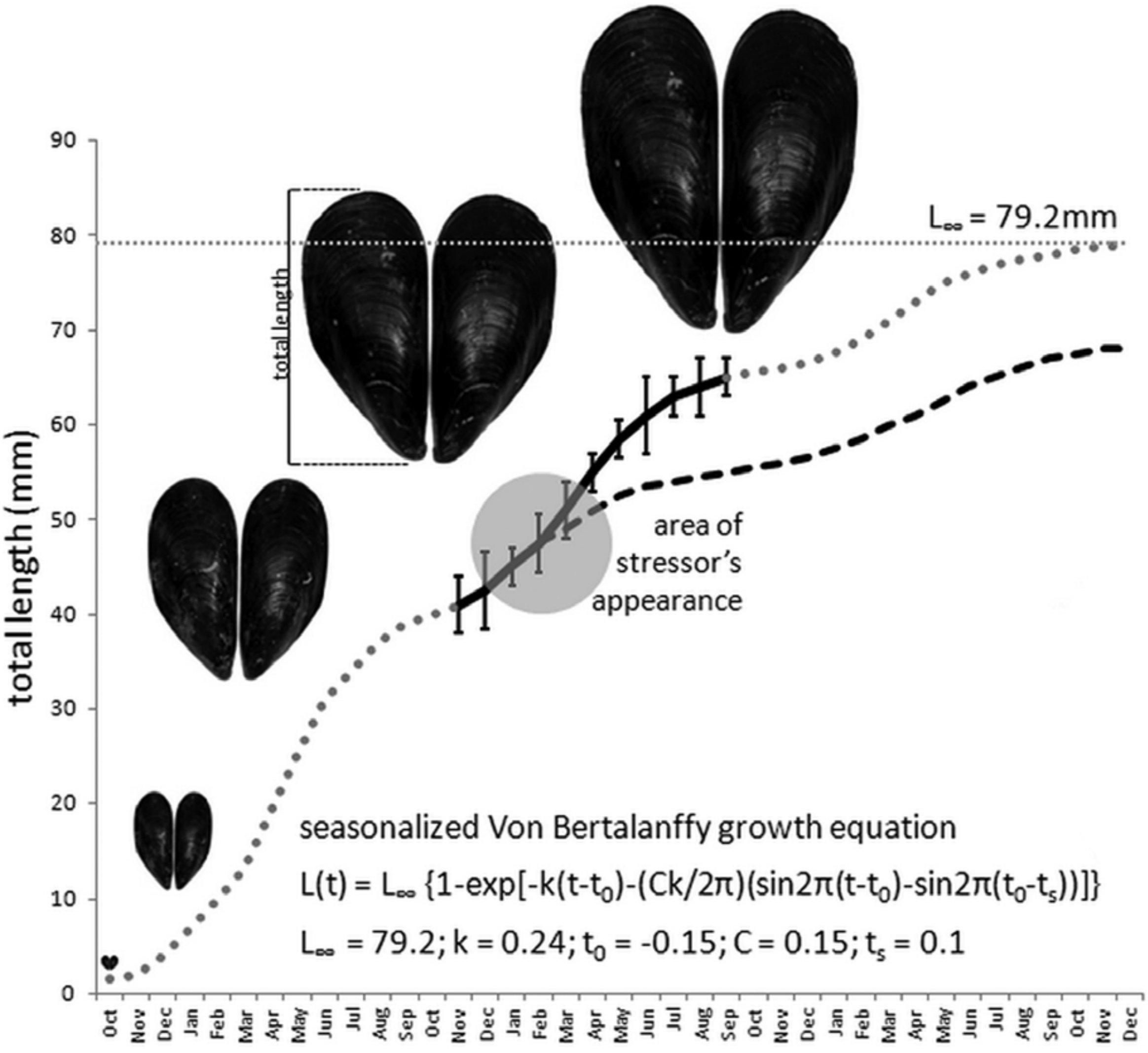

Fig. 3 Simulated natural growth rate (gray points and black continuous lines) and simulated growth rate after the appearance of a stressor (black dotted line). In alphabetical order: C = amplitude of the curve (providing an estimate of the influence of seasonality on growth trends); k = curvature parameter (describing how quickly individuals approach asymptotic length); L_∞ = asymptotic length (theoretical max length); t_0 = initial condition parameter (time when the specimens have zero length); t_s = summer point (referring to the onset of the first seasonal oscillation relative to t = 0).

Panel S1

When age and size may be associated, we can express their relationship by the von Bertalanffy equation:

$L(t) = L_\infty\{1-\exp[-k(t-t0)]\}$

where L_∞ is the asymptotic length (theoretical max length), k the curvature parameter (describing how fast individuals approach the asymptotic length), and t_0 the initial condition parameter (time when specimens have zero length). This growth model has been showed extensively suitable for the assessment of a large number of marine animals both vertebrate and non. Once we obtain L_∞, and t_0, we may evaluate further population properties as well:

1) the total mortality index (the sum of natural mortality and the mortality due to fishing) by the Powell-Wetherall Plot equation, that computes the asymptotic length and the ratio between the mortality coefficient and the curvature parameter (Z/k);

2) the natural mortality (M) by the formula:

$\log M = - 0.0066 - 0.279\log L_\infty + 0.6543\log k + 0.463\log T$

where T is the mean environmental temperature;

3) the fishing mortality (F) subtracting M from Z;

4) the expected mean life-time and the longevity:

$t_{1/2} = \Sigma\{[n(t)*t]\}/N$; $t_{max} = (3/k) + t_0$

respectively, where n(t) is the number of individuals at the time t and N is the total number of individuals.
For more details on the stock assessment concepts and tools, see Sparre and Venema (1996).

Fishery biology traditionally uses the von Bertalanffy growth model equation to determine population properties such as mortality rates and decay model parameters (see Panel S1). Length-frequency data entry and manipulation can be used to create an information flow that enables assessments of capture probability and mortality, as well as evaluations of stock damage via virtual population analysis. These finding can be used to create predictive models.

The evaluation of population structure and dynamics is essential in the management of aquatic living resources because this information can be used to evaluate population status. Such evaluations are useful for in situ monitoring of population status and could have roles in introduction programs and in monitoring reintroduced individuals during restocking. In fact, population dynamics are strongly associated with biological processes and thus the adaptation of a species to its habitat. Hence, environmental changes (such as those mediated by humans) can stress a population and induce alterations in valve shape and integrity (see also Bressan *et al*., 2014) and, consequently, dynamics (Fig. 3). Such changes can have ecosystem-level consequences when stressed species show a some kind of relevance for the energy flow in an ecosystem.

The threat of pollution to mussels is more evident when we consider that valve malformations may also compromise the actions of the mantle as a gonad. This can cause fitness reduction and consequent population reduction. In turn, the reduced mussel biomass may affect other species because many organisms, including economically important species, feed on *M. galloprovincialis* in both the larval and adult phases.

Perspectives

Monitoring programs exploiting a part of a cenosis (*e.g*., benthic taxa) often provide useful information on contamination than that obtained using populations and species. The two latter difficulties supply information concerning the relationships between causes and effects at level of community or ecosystem. Indeed, several investigations on the effects of environmental human-mediated constrains on benthic organisms are developed (Parker *et al*., 2011, Range *et al*., 2011; Talmage and Gobler, 2011; Bressan *et al*., 2014), but direct effects at food-web and ecosystem levels are never provided. This is due to the strong difficulties in extrapolating general proxies from populations or species, since on one hand, many synergic (natural or anthropogenic) drivers may act at higher levels of the hierarchical biological organization; on the other hand, higher levels show more and more complex processes of resistance and resilience. Therefore, in this proposal, we focused on methods of highlighting the effects of pollutants on biological descriptors that are easy to survey and may be useful as early warning systems for the whole ecosystem health. Here, we proposed integrated tools for using natural resources as monitors to survey and mitigate the impact of pollutants on the environment and explored the use of predictive models to forecast the effects of contaminants.

To fill this gap, future risks may be evaluated exploiting multi-level approaches in long-term experiments and exposure to multiple stressors. Further research in the lab and in the field should be initiated and funded to better understand (1) the mechanisms, advantages and drawbacks of the integrated approach and (2) the criteria by which to select an appropriate strategy of management and/or preservation.

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