

Review

Assessments of lysosomal membrane responses to stresses with neutral red retention assay and its potential application in the improvement of bivalve aquaculture

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In marine bivalves, it has been demonstrated that their lysosomal membrane stability are very susceptible to many internal and external environmental changes and this physiological response can be quantified by the neutral red retention (NRR) assay. This assay has been applied in many recent studies in the areas of environmental monitoring with bivalve species and physiological responses of farmed bivalve species to changes in environmental parameters (stressors) and handling practices. The published results show that lysosomal membrane stabilities respond to these changes in a dose dependent manner and are determined by the stress types, their levels/dosages and exposure durations. In addition, the recovery of lysosomal membrane stability after stress treatment is likely to be size/age dependent, with smaller/younger bivalves recovering quicker than bigger/older individuals. These indicate that the NRR assay could be used by the aquaculturists and/or farmers to: 1) monitor the bivalve health status within the environments where the animals are farmed and 2) improve not only the efficiency of bivalve farming practices but also the performances of the bivalves farmed.

Key words: Stress, lysosomal membrane stability, neutral red retention assay, bivalves, aquaculture.

INTRODUCTION

Nowadays, the aquaculture industry in the world is experiencing more challenges than ever before due to the increased impacts from climate changes, substantial expansion in the areas utilised for cultivation, deterioration in environmental conditions, increased frequency of disease outbreaks and suboptimal farming practices. All these could impose serious extra stresses on the animals reared, thus affecting their survival, growth, health, nutrition value and welfare, and eventually hampering the development of aquaculture industries. In bivalves, for example, abnormal mortality has affected the industry heavily in a few regions in the world (Cheney et al., 1998; Burge et al., 2005; Geret et al., 2004; Samain et al., 2004; Cotter et al., 2010), which is confirmed to be resultant of a complex interaction between multiple factors including both internal and

external environmental changes, and the presence of pathogens. It is also anticipated that the negative impacts of these factors could be minimised or even eliminated if they could be predicted and quantified by method(s) that is cost-effective and easy to apply.

Lysosomes are dynamic, polymorphic and hydrolytic enzyme-containing organelles that receive and degrade macromolecules from the secretory, endocytic, autophagic and phagocytic membrane-trafficking pathways (Luzio et al., 2007). They play an important role in the detoxification and defence in marine organisms (Moore, 1980; Lowe et al., 1995a, b). As these functions are a membrane-dependent process, the stability of lysosomal membrane could then be used to determine their efficiency in performing these functions. The neutral red retention (NRR) assay has been developed base on this principle (Lowe et al., 1992, 1995a, b). Neutral red is a lipophilic chemical and can passively diffuse through the cell membrane (Lowe et al., 1992). The efficiency of neutral red retention depends on the pH of the lysosome

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and the efficiency of its membrane bound proton pump (Seglen, 1983) that maintains the acid condition of lysosome (Ohkuma et al., 1982). Therefore, lysosomes in unstressed cells can retain the neutral red for a long duration after uptake. However, when the lysosomal membrane, or possibly the H⁺ ion pump is destabilized, the neutral red will leak into the cytosol of the cell much more quickly (Moore, 1980; Lowe et al., 1992). NRR assay is commonly used as a bio-marker to monitor the health of marine environments (Da Ros et al., 2002; Martinez-Gomez et al., 2008; Franzellitti et al., 2010) and to evaluate the effects of different stressors on many marine bivalves, especially oysters (*Crassostrea gigas* and *Ostrea edulis*) and mussels (*Mytilus galloprovincialis* and *Mytilus edulis*) (Lowe et al., 1995a, b; Hauton et al., 1998, 2001; Cho and Jeong, 2005; Mamaca et al., 2005; Zhang et al., 2006; Guidi et al., 2010). Luzio et al. (2007) and Moore et al. (2008) reviewed lysosomal characteristics and their physiological and immunological functions. The present review summarises the recent findings that could potentially be used to improve practices in bivalve aquacultures and thus aquaculture productions, with primary focuses on: 1) the definition and category of stresses; 2) stress impacts on lysosomal membrane stabilities and 3) the potential application of NRR assay in the improvement of shellfish aquaculture.

STRESS

Stress has been defined as any stimulus that disturbs normal functions in an organism, whereas any reaction in the organism to this stimulus is called the stress response (Bayne, 1985). Stresses can be generally divided into two categories in aquaculture: environmental and artificial stresses. Environmental stress includes water parameter change (water temperature, salinity and pH), chemical pollutant (pesticides, heavy metals and hydrocarbon), physiological (such as reproduction and starvation) and pathogenic (bacteria, viruses and parasites) stresses. More attention has been focused on these stress effects on aquaculture species than ever before as they will be further enhanced by the anticipated global climate changes. Artificial stress, on the other hand, includes the man made stresses in aquaculture practices, such as grading, debyssing, washing and declumping. Therefore, understanding of artificial stresses on cultured marine bivalves would be important, as environmental stresses that could be directly applied to improve the farming practices and thus the performance of farmed organisms.

CORRELATION BETWEEN STRESSES AND LYSOSOMAL MEMBRANE STABILITY

Chemical pollutant stresses

Chemical pollution has become a great challenge to

bivalve farming practitioners as well as the organisms farmed, thus affecting the healthy development of aquaculture industry. In addition, these pollutants can also be accumulated in marine organisms (Andrea et al., 2007; Li et al., 2010; Jia et al., 2010; Lukashov, 2010). Although detailed mechanisms remain largely unknown, environmental pollutants have been reported to affect reproduction (Choy et al., 2007) and embryonic development (Rosen et al., 2008), and damage DNA structures (Pan et al., 2008), gene expression (Ivanina et al., 2010) and signalling between cells (Martin-Diaz et al., 2009) in marine bivalves. However, how to effectively and efficiently assess the impacts of chemical pollutants on cultivated marine bivalves presents a challenge to the science community. The response of lysosomes to these pollutants might provide a new approach to address this as they involve in the detoxification processes. Therefore, the development of NRR assay, which assesses the integrity of lysosome membrane, has enhanced the capacities in this research area and has been applied in many investigations with marine bivalves, including studies in both environmental toxicology and environmental pollution. Results from these studies have provided important information to bivalve aquaculture, although most of these studies were not motivated by this direction.

Chemical pollutants destabilized lysosomal membranes of marine bivalves in an exposure duration and/or dose dependent manner. For example, the stability of lysosomal membrane of hemocytes in the mussel, *M. edulis* was significantly reduced after 30 min exposure to 10 ppm xenobiotics (Moore et al., 1996), suggesting that xenobiotics at 10 ppm level has probably affected the physiological and health conditions in this species after the exposure. Heavy metals are other kinds of chemical pollutants having significantly affected offshore cultivated bivalves in many regions.

Furthermore, different heavy metals have different effects on the stability of lysosomal membrane in marine bivalves as well. In the study of Brown et al. (2004), the stability of lysosomal membrane was significantly reduced after seven days exposure to Cu at the concentration of 6.1 µg l⁻¹. At the same concentration, on the other hand, the membrane stability was less affected by Cd (Brown et al., 2004). Mixing two or more different heavy metals has additive effects on lysosomal membrane destabilization (Bolognesi et al., 1999). The effect of hydrocarbons on lysosomal membrane disturbance depends on their types and doses too. Canesi et al. (2002) studied effects of four polychlorinated biphenyls congeners at two different concentrations on lysosomal membrane stability in the mussel, *M. galloprovincialis*. They found that three of them at high concentration (10 µg/ml) had significant effects on the lysosomal membrane stability whereas none had significant effect at low concentration (1 µg/ml) (Canesi et al., 2002). This pinpoints the importance of dose measurement of pollutants in the regions surrounding

the species farmed. Beside the above-mentioned pollutants, the impacts of other chemicals on the lysosomal membrane stability have also been reported (Dickhut and Gustafson, 1995).

Water parameter change stresses

Water temperature is one of the common and important environmental factors affecting the physiological conditions of marine bivalves. NRR assay has been confirmed to be a simple and effective method to study the optimal water temperature ranges for marine organisms. A water temperature range of 14 to 16°C was found to be the optimal temperature for maintaining hemocyte lysosomal stability in both the European flat oyster *Ostrea edulis* (Hauton et al., 1998) and the Pacific oyster *C. gigas* (Zhang et al., 2006). Range lower or higher than this could reduce the stability of lysosomal membrane substantially in these species. In Pacific oysters, for example, the lysosomal membrane stability decreased significantly when water temperature decreased to 5°C (Zhang et al., 2006). Similar results were also reported in other species such as *M. edulis* (Camus et al., 2000). Both gradual and rapid changes in water temperature (from 15 to 5 or 25°C) significantly reduced hemocyte lysosomal membrane stability in *C. gigas* (Zhang et al., 2006). However, the lysosomal membrane stability was not influenced by the speed at which water temperature was changed (Zhang et al., 2006). In surf clam *Macra veneriformis*, Yu et al. (2009) reported a similar phenomenon that NRR times significantly decreased at 10 and 30°C in comparison with that at 20°C. On a seasonal scale, Ringwood et al. (2002) reported that lysosomal destabilization rates tend to be higher during the winter at both clean and polluted sites. All these studies have enriched our understanding of water temperature change effects on the physiological/immunological conditions of cultured organisms.

The durations required for lysosomal membrane integrity recovery were longer than those for destabilization in marine mollusks, which is strongly supported by the study of Zhang et al. (2006) in *C. gigas*. According to their study, when oysters were transferred from 15 to 5 or 25°C water, the lysosomal membrane stability was reduced to the lowest level within 3 h. In comparison, it took five days for the lysosomal stability to recover when oysters were transferred in the opposite direction (from 5 or 25°C water to 15°C water). In addition, Song et al. (2007a) also found that large oysters required a longer duration to recover their lysosomal integrity than small individuals when the water temperature changed from 5 or 25 to 15°C in this species. This suggests that large marine bivalves might be more vulnerable to water temperature changes.

A suitable salinity range is also essential for the perfor-

mances (such as survival, growth and health) of cultivated marine bivalves. Coughlan et al. (2009) found that salinity had a significant effect on the stability of lysosomal membrane in the Manila clam *Ruditapes philippinarum*. Similarly, Hauton et al. (1998) reported that 32‰ was the optimal salinity for maintaining the lysosomal membrane stability in *O. edulis*. In their study, these lipid membranes were less stable at low salinities (16 and 19‰).

Air exposure, a common experience in Pacific oysters that occur or are farmed in the intertidal regions, significantly impaired the lysosomal membrane stability in this species (Zhang et al., 2006). Moreover, exposure to lower air of temperatures caused less damage to lysosomal membrane than higher temperatures (Zhang et al., 2006). After air temperature, the lysosomal membrane integrity of the large oysters recovered at a slower rate than did the small individuals (Song et al., 2007a).

Physiological stress

Spawning is an important physiological stressor in aquaculture bivalve species, which reduced the lysosomal membrane stability in *C. gigas* significantly (Cho and Jeong, 2005). They attributed this phenomenon to the energetic cost of spawning event, which, in turn affects the state of cellular health. These could result in the increased susceptibility to the infection of parasites and pathogens, thus mortalities in this species (Cho and Jeong, 2005). Song et al. (2007b) further extended the study over the period from gonad development to after spawning recovery. Their study showed that prior to spawning, the lysosomal membrane stability of oysters fed with a higher level of microalgae were significantly reduced in comparison with those fed with a level of microalgae which is enough to maintain their conditions during this period. This result suggests the decreased lysosomal membrane stability during this period might be caused by gametogenesis, a nutritional imbalance or a combination of both (Song et al., 2007b). Their results also showed that spawning significantly impaired lysosomal membrane stability. After spawning, the lysosomal membrane stability of hemocytes remained at the lowest levels for at least 12 days before recovering to the levels corresponding with the water temperature. Song et al. (2007b) also found that the smaller oysters recovered from these reproduction/spawning stresses were significantly faster than the larger animals.

Pathogenic stresses

Although only a few reports are available, effects of pathogens on lysosomal membrane stability of marine bivalves are shown to be both animal and pathogen dependant, indicating a complex interaction between

them. Hauton et al. (2001) studied the effects of *Listonella anguillarum* inoculation on the hemocyte lysosomal membrane stability in the European flat oyster *O. edulis*, Pacific oyster *C. gigas* and scallop *Pecten maximus*. According to their study, lysosomal membrane stability was reduced in the presence of bacteria for all these three species. Among them, *C. gigas* was the most sensitive species to the bacterial presence and showed significant reduction in lysosomal membrane stability in all the experimental water temperatures (Hauton et al., 2001). In addition, Ciacci et al. (2009) also showed that different bacteria have different impacts on the stability of lysosomal membrane in the mussel *Mytilus galloprovincialis*. Further studies are needed to investigate the synergistic effects of multiple pathogens on marine mollusks farmed.

HANDLING STRESSES IN AQUACULTURE PRACTICES

Artificial stress is the main area in which NRR assay can really provide information to further improve the aquaculture practices and at the same time minimise the potential negative impact on the performances of the bivalves farmed. As Dimitriadis and Koukouzika (2003) pointed out, care should be taken in handling farmed marine bivalves. As compared to all the other stresses, which cannot be managed effectively, if any, as they are mainly controlled by the changes in the natural environments, artificial stresses, like handling, are add-on practices developed for the species farmed. The establishment of aquaculture industry is a milestone in the evolution of agri-business, which has been greatly promoted in many coastal countries including both developing and developed countries. Techniques developed for one species is then applied to other species, resulting in the commercialisation of many new species over the last few decades. However, investigations on the impacts of farming practices on the physiological conditions and welfare of bivalves farmed cannot keep the pace of industry development. The application of NRR assay would accelerate our understanding in the field and has been started in recent years

Although, only a few reports are available, the results from NRR assays have clearly demonstrated the effects of handling on bivalves farmed. Zhang and Li (2006) reported that the oysters (*C. gigas*) responded to grading in a duration-dependent manner, that is, those oysters undergoing longer durations of grading expressed lower NRR times. This suggests that farmers should decrease handling time as much as possible in aquaculture managements. In addition, shortening handling time should benefit not only the physiological conditions of marine mollusks farmed but also their recover processes. This hypothesis is strongly supported by the research

conducted by Zhang and Li (2006). In their study, the NRR times in the oysters graded for shorter periods returned to the control level more quickly than those graded for longer durations. Different artificial stresses might have different effects on marine bivalves. The study of Harding et al. (2004) is a good example. They studied the effects of debysing, washing and declumping on the lysosomal membrane stability of hemocytes in mussels (*Mytilus* spp.) and found that debysing induced significant negative effects on the lysosomal membrane stability of mussels in all the seasons studied. Washing and declumping, on the other hand, had, if any, no significant effect on the stability of lysosomal stability in this species (Harding et al., 2004). These experimental results would therefore provide where the attention should be paid to further improve the performances of this species over the harvesting process. To optimize aquaculture systems and operations for marine bivalve aquaculture, it is anticipated that more detailed investigations in this area would certainly be required.

CONCLUSION

Stress is the result of a complex interaction among environment, marine organisms and pathogens, and has contributed to abnormal mortalities in farmed bivalves. It is therefore essential and urgent to understand stresses and their relationships with the physiological and immunological conditions in marine bivalves. In this paper, we reviewed the application of NRR assay to evaluate effects of both environmental and artificial stresses on the lysosomal membrane stability in marine bivalves and their potential implication for improvement of aquaculture practices.

Cultivated marine bivalves always experience stresses from multiple factors and stresses resulting from multiple factors are normally more severe than those from single factors. For example, the threat imposed by the bacterium *L. anguillarum* to the bivalve hosts was greater at 15°C than at other temperatures (Hauton et al., 2001), indicating that both water temperature and bacterium have played an important role in this process. Furthermore, both environmental and artificial stressors can react collectively on the stability of lysosomal membrane in marine bivalves. In *C. gigas*, for example, Zhang and Li (2006) found that after exposure to a simulated grading, the lysosomal membrane stabilities in oysters starved for 42 days differed significantly from those that were fed over this period.

It should be noted that in addition to NRR assay, there are also other methods that could be developed to measure the stability of lysosomal membrane in marine bivalves, including lysosomal latency test (LLT) and neutral red uptake (NRU) assay. However, NRR assay has been approved as the most cost-effective assay

among them.

In conclusion, NRR assay could be used to: 1) monitor the performances and health status in farmed marine bivalves, with the support of other assays if available and practical and 2) improve various aspects of bivalve aquaculture managements, especially in the areas of farming practices.

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