Marine Pollution Bulletin 58 (2009) 1680–1684

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

The effects of salinity on the Manila clam (*Ruditapes philippinarum*) using the neutral red retention assay with adapted physiological saline solutions

B.M. Coughlan [a,b,*], G.A. Moroney [b], F.N.A.M. van Pelt [a,c], N.M. O'Brien [a,d], J. Davenport [a,b], J. O'Halloran [a,b]

[a] Environmental Research Institute, University College Cork, Cork, Ireland
[b] Department of Zoology, Ecology and Plant Science, University College Cork, Cork, Ireland
[c] Department of Pharmacology and Therapeutics, University College Cork, Cork, Ireland
[d] Department of Food and Nutritional Sciences, University College Cork, Cork, Ireland

ARTICLE INFO

Keywords:
Biomarkers
Clams
Lysosomes
Neutral red retention
Salinity
Physiological saline solutions

ABSTRACT

This study investigated the internal osmotic regulatory capabilities of the Manila clam (*Ruditapes philippinarum*) following *in vivo* exposure to a range of salinities. A second objective was to measure the health status of the Manila clam following exposure to different salinities using the neutral red retention (NRR) assay, and to compare results using a range of physiological saline solutions (PSS). On exposure to seawater of differing salinities, the Manila clam followed a pattern of an osmoconformer, although they seemed to partially regulate their circulatory haemolytic fluids to be hyperosmotic to the surrounding aqueous environment. Significant differences were found when different PSS were used, emphasizing the importance of using a suitable PSS to reduce additional osmotic stress. Using PSS in the NRR assay that do not exert additional damage to lysosomal membrane integrity will help to more accurately quantify the effects of exposure to pollutants on the organism(s) under investigation.

1. Introduction

There is an ever increasing desire to use biochemical, cellular, and physiological biomarkers, to investigate the detrimental effects pollutants are having on aquatic organisms (Depledge et al., 1995). Biomarkers can measure biochemical, cellular, physiological or behavioural changes in tissues, body fluid samples or at the level of whole organisms, resulting from environmental and/or chemical exposure (Depledge, 1993).

Disruption to metabolic and physiological processes leading to adverse effects being expressed at the individual, population and community levels of biological organization, have been linked to molecular damage and cell injury resulting from pollution uptake, biotransformation and radical generation (Slater, 1979; Bayne et al., 1988; Maddox, 1998; Moore, 2002; Brown et al., 2004; Duquesne et al., 2004). Molecular and cytological biomarkers have a high sensitivity and specificity to stressors including pollutants, and can act as 'early warning stress biomarkers' (McCarthy and Shugart, 1990; Moore et al., 1996; Nicholson and Lam, 2005). A well established sentinel cytological biomarker is the assessment of lysosomal destabilization, which has been shown to correlate closely to stress (Moore, 1985; Regoli, 1992; Nicholson, 1999a; Lowe and Fossato, 2000; Moore et al., 2006a,b).

A widely used biomarker, measuring lysosomal membrane integrity that mirrors progressive lysosomal destabilization, is the neutral red retention (NRR) assay as described by Lowe et al. (1995). This assay involves the extraction of haemolymph into an equal volume of physiological saline solution (PSS) as described by Peek and Gabbott (1989), to which a neutral red dye is added and the rate of leakage of the dye from the lysosomes is measured. Since the development of the assay, it has been successfully used as a cost-effective and sensitive ecotoxicological tool, investigating the effects of various stressors on aquatic organisms mostly in marine bivalves (Moore et al., 1996; Cheung et al., 1998; Ringwood et al., 1998; Nicholson, 1999b; Fernley et al., 2000; Livingstone et al., 2000; Wedderburn et al., 2000; Brown et al., 2004; Galloway et al., 2004; Martins et al., 2005; Nigro et al., 2006).

Bivalves make ideal aquatic bioindicators because they have a wide geographical distribution, sedentary behaviour, ease of sampling, tolerance to a wide range of environmental conditions, and readily sequester contaminants (Cajaraville et al., 2000; Lam and Wu, 2003). However, when choosing a suitable bioindicator to investigate pollutant exposure in bays and estuaries, it is important to take into account the additional factor of changing salinities commonly occurring in such ecosystems. The ebb and flood of the tide, combined with fresh water inputs from rivers and tributaries can dramatically alter the salinity of water. For this reason, it would be beneficial to choose a species that is known to inhabit aquatic environments of varying salinity and have appropriate physiological adaptations.

* Corresponding author. Address: Environmental Research Institute, University College Cork, Cork, Ireland. Tel.: +353 21 490 4596; fax: +353 21 490 4664.
E-mail address: b.coughlan@student.ucc.ie (B.M. Coughlan).

0025-326X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.marpolbul.2009.06.020

The euryhaline bivalve Manila clam (*Ruditapes philippinarum*) makes an ideal candidate as biomonitor or ecotoxicological test organism, as it is found world-wide and is currently being commercially farmed in China, Japan, Canada, America, Italy, France, the UK and Ireland (Gosling and Nolan, 1989; Iversen and Hale, 1992; Elston et al., 2001; Hideo and Ryo, 2003; Jensen et al., 2004; Soudant et al., 2004; Melia and Gatto, 2005; Zhang and Yan, 2006). The Manila clam has been farmed in salinities ranging from 20‰ to full strength seawater, although the recommended optimum range for farming is between 20‰ and 30‰. Recent studies of the salinity range of the Manila clam report that they can maintain normal metabolic activities, following long term exposure to full strength seawater, down to 15‰ (Kim et al., 2001; Elston et al., 2003). However, below 15‰, histological analysis revealed clams exhibiting physiological and morphological abnormalities with ensuing mortalities. In addition, several studies have shown clams to be sensitive bioindicators when exposed to chronic water/sediment pollution (Blasco and Puppo, 1999; Nasci et al., 1999; Byrne and O'Halloran, 2000, 2001; Coughlan et al., 2002; Tay et al., 2003; Werner et al., 2004; Martins et al., 2005; Hartl et al., 2006).

This study involved two investigations, using the Manila clam, one aimed at examining whether the Manila clam is a true osmoconformer. The second objective was to specifically examine the effects of using adapted PSS with different salinities when performing the NRR assay.

2. Materials and methods

2.1. Clams

Manila clams, *R. philippinarum*, of known history (3+ age class) were obtained from Cartron Point Shellfish Ltd., a commercial hatchery on the west coast of Ireland. The animals were transported to the laboratory and acclimated in aerated seawater (35‰) in a stock tank prior to exposure to different experimental investigations. During this time, the clams were fed with Phytoplex (a composite of laboratory cultured natural marine planktons supplied by Fish Antics, Dublin, Ireland). The mean shell length and weight of the clams at the start of this study were 40 ± 3 mm and 18 ± 2 g, respectively.

2.2. Substratum

Horticultural sand (Glenview Horticultural Sand, Woodies, Cork, Ireland), was used as a source of uncontaminated soft substratum for the clams to bury. The sand used was repeatedly pre-washed with seawater and passed through a 1 mm sieve to remove any large debris.

2.3. Examination of euryhaline capabilities

2.3.1. Experimental and exposure regime

An *in vivo* experiment was conducted in a controlled environment (15 ± 1 °C; 12 h light/dark cycle). On arrival, the clams were allowed 48 h to acclimatise at 35‰ in a stock tank, to reduce any stress resulting from transportation and handling. The sand was divided into 4.5 kg aliquots, each aliquot was then thoroughly homogenised with 10 L of seawater (35‰), placed into six separate 15 L pre-cleaned glass aquariums and allowed to settle. Once the sand had settled, 20 clams were randomly selected from the stock population and gently placed into each aquarium for 24 h.

Initially, 10 clams were randomly chosen from the stock population. Each clam was partially prised open, a sample of haemolymph was extracted from the posterior adductor muscle using a 21 gauge needle syringe, and the salinity of the haemolymph was assessed using a handheld Automatic Temperature Compensating Refractometer (Tropical Marine Centre Ltd., Hertfordshire, UK). This is a relatively crude method of assessing haemolymph osmolarity (its accuracy is about 0.5‰ (equivalent to about 15 mOsm)), but quick and convenient. In addition, the salinities of all six aquaria were reduced by 3‰ using de-chlorinated water. Thereafter, the seawater in the aquaria was further reduced by 3‰ on subsequent days, until salinities of 29‰, 26‰ and 20‰ had been achieved (allowing duplicates per treatment). Once each of the desired seawater salinities in the aquaria had been reached, it was maintained for 96 h to allow the clams to acclimatise to the decreased salinity. After 96 h, 10 clams (five from each duplicate) exposed to each salinity were removed, a sample of haemolymph extracted from each individual and assessed using the refractometer, as above.

The seawater in the aquaria was continuously aerated and throughout the experiment the clams were fed with Phytoplex every 2 days.

2.4. Examination of neutral red retention

2.4.1. Lysosomal stability

The lysosomal stability of the haemocytes was assessed using the NRR assay as described by Lowe et al. (1995), using adapted PSS. The clams were partially prised open, and haemolymph extracted from the posterior adductor muscle using a 21 gauge needle syringe into an equal volume of the respectively chosen physiological saline solution, and transferred into a 1.7 ml presiliconised eppendorf tube. The haemolymph/physiological saline mixture (40 μl) was pipetted onto a glass slide pre-coated with a poly-L-lysine solution, and incubated for 15 min in a light proof humidity chamber to allow the cells to adhere. The excess solution was then gently tipped off, 40 μl of neutral red dye was added and a coverslip applied, before being replaced in a light proof humidity chamber for a further 15 min. The retention of the dye within the lysosomes in the cells was recorded every 15 min for to the first hour, and then at 30 min intervals until over 50% of the cells demonstrated leakage of the neutral red dye from the lysosomes and vacuole formation was recorded.

2.4.2. Physiological saline solutions

Three specific bivalve PSS were used in this study with salinities of 38.5‰, 32.5‰ and 26.5‰ (Table 1), and each PSS was adjusted to a pH of 7.36. These salinities were chosen to ensure that PSS 1, PSS 2 and PSS 3 salinities were approximately 0.5–1‰ above that of the haemolymph, to avoid inflicting hypotonic stress that could cause the cells to swell and rupture.

2.4.3. Experimental and exposure regime

The *in vivo* experiment was established as above. Initially, the salinity in two aquaria was maintained at 35‰ whilst the remaining four aquaria were reduced by 3‰ using de-chlorinated water. Thereafter, the seawater in the four aquaria was further reduced by 3‰ on subsequent days, until salinities of 29‰ and 23‰ had been achieved (allowing duplicates per salinity). Once the desired salinity of the seawater in the aquaria had been reached, it was maintained for 96 h to allow the clams to acclimatise to the adjustment in salinity. After 96 h, nine clams were randomly selected from the aquarium, their haemolymph extracted and the NRR assay was conducted.

The addition of each group of clams and duplicates, with corresponding adjustment of salinity for each of the different seawater salinities under investigation (35‰, 29‰ and 23‰), was staggered. This was to ensure uniformity in the duration of exposure of the

Table 1
The chemical composition and salinities of physiological saline solutions utilised in the NRR assay, as described in this study and by Peek and Gabbott (1989), Dailianis et al. (2003), Brown et al. (2004) and Marchi et al. (2004).

Papers/studies		Salinity (‰)	Chemical composition (molarity)						
			NaCl	$MgSO_4$	$CaCl_2$	KCL	HEPES	EDTA	95% O_2 5% CO_2
Peek and Gabbott (1989)		?	0.436	0.053	0.01	0.01	0.02	0.005	Gassed for 10 min
Brown et al. (2004)		41	0.4	0.1	0.01	0.01	0.02	0	0
Dailianis et al. (2003)		46	0.436	0.11	0.01	0.01	0.02	0	0
Marchi et al. (2004)		38	0.436	0.053	0.01	0.01	0.02	0	0
Current study	PSS 1	38.5	0.4	0.071	0.01	0.01	0.02	0	0
	PSS 2	32.5	0.325	0.0577	0.01	0.01	0.02	0	0
	PSS 3	26.5	0.25	0.0444	0.01	0.01	0.02	0	0

Table 2
Statistical analyses of neutral red retention times of haemolytic lysosomes isolated from the Manila clam (*R. philippinarum*), following exposure to seawater of varying salinities and using different physiological saline solutions when extracting the haemolymph for the NRR assay.

			Exposure time to seawater						
			Day 0		Day 4				
			35‰		35‰	29‰		23‰	
			PSS 1	PSS 2	PSS 1	PSS 1	PSS 2	PSS 1	PSS 3
Day 0	35‰	PSS 1		***	N/S	***	N/S	***	***
		PSS 2			***	***	***	N/S	***
Day 4	35‰	PSS 1				***	N/S	***	***
	29‰	PSS 1					***	***	*
		PSS 2						***	***
	23‰	PSS 1							***
		PSS 3							

‰: Seawater salinity (parts per thousand); PSS: physiological saline solution; N/S: no significant difference; (*): significant difference ($P < 0.05$); (***): significant difference ($P < 0.001$).

clams to the different seawater salinities (96 h), and allow sufficient sample size to be available for the NRR assay.

During the initial 96 h of acclimatisation to the salinities in the aquaria, two studies were conducted (both in duplicate). In the first study, nine clams were randomly selected from the stock population (35‰), and a NRR assay was performed using PSS 1 (control), this was repeated the following day (duplicate). In the second study, this method was repeated but PSS 2 was used.

2.5. Statistical analysis

The data obtained were tested for normality (Shapiro–Wilks), but the data was found not to conform to normality. Therefore, all data were analysed by non-parametric statistics.

3. Results

3.1. Haemolymph salinities

The salinity of the haemolymph was found to be consistently 2.5 ± 0.5‰ higher than the ambient salinity. This corresponds to a hyperosmocity of around 75 ± 15 mOsm.

3.2. Lysosomal stability

No significant difference was found between duplicates, so duplicate data were pooled and results of statistical analyses are presented in Table 2.

4. Discussion

This study examined whether the Manila clam that inhabits marine environments with differing salinity gradients, was a true osmoconformer. To the authors knowledge there have been no published studies showing whether the Manila clam has specific abilities to regulate the passage of water through membranes, in relation to any external salinity changes. We report that, although the Manila clam did follow the pattern of an osmoconformer, they also showed evidence of partially osmoregulating their haemolymph to be approximately 2.5‰ (equivalent to about 75 mOsm) above that of the aquarium seawater. Euryhaline marine osmoconformers show a slight degree of hyperosmocity to the environment due to the Donnan Equilibrium (caused by the presence of impermeant anions within the animal), but not to the extent of 75 mOsm. This degree of hyperosmocity is compatible with the recently-expressed view that some osmoconformers can regulate their cellular osmotic pressure by using a variety of organic osmolytes (Yancey, 2005).

Previously described as 'compatible solutes' (Brown and Simpson, 1972), organic osmolytes have been extensively reviewed (Yancey et al., 1982; Yancey, 2001), and found in many species (including clams *Saxidomus giganteus* and the *Calyptogena* spp.) to have cytoprotective properties (Yancey, 2005). Yancey (2005) suggests that by increasing or decreasing the levels of organic osmolytes such as taurine, betaine and alpha-amino acids in response to osmotic changes in the aquatic environment, it appears that organic osmolytes have unique properties such as protecting metabolic reactions and counteracting destabilizing forces on macromolecules, which help prevent cellular damage from occurring. The results from the present study suggest that the Manila clam may also be using organic osmolytes, as reported recently in other species of clams (Yancey, 2005).

Since the development of the NRR assay it has become widely used in many studies and projects, such as the pan-European EU funded research programme titled "Biological Effects of Environmental Pollution in Marine Coastal Ecosystems" (BEEP) (ICES, WGBEC, 2004). However, to the author's knowledge all studies have used the same or an adapted version of the PSS as that described by Peek and Gabbott (1989), throughout the study irrespective of investigating different sites and/or aquatic environments. Yet our study showed that the salinity of the euryhaline Manila clam's internal fluids varied in accordance with the surrounding seawater. If the NRR assay utilising the same PSS was to be used *in situ* or *in vivo* studies of different salinities, in theory extra osmotic stress could be imposed upon the haemolymph cells. Use of a hypersaline medium which has a total amount of solutes in solution that is greater than that in the haemolymph cells, could result in movement of water out of the haemolymph cells and cause damage to internal cellular metabolic processes. In contrast, a hyposaline medium would result in the influx of water into the

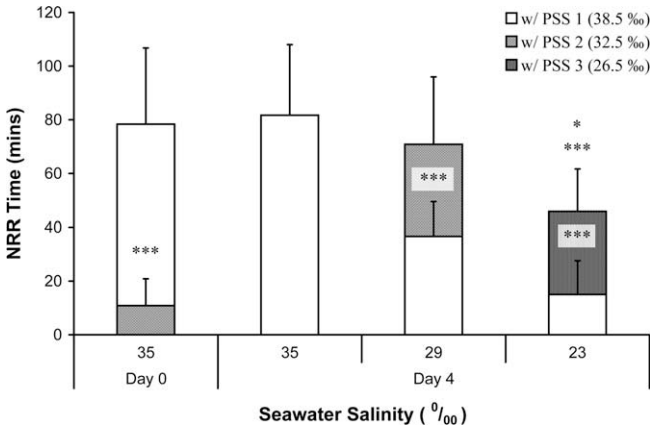

Fig. 1. The neutral red retention time of haemolymph cells isolated from the Manila clam (*R. philippinarum*), following exposure to seawater of varying salinities and using different physiological saline solutions when extracting the haemolymph for the NRR assay; mean ± SD; $n = 18$; (∗): significant difference ($P < 0.05$); (∗∗∗): significant difference ($P < 0.001$).

haemolymph cells and cellular organelles, causing swelling and rupture of cellular components and ultimately the cells. This would cause additional lysosomal membrane destabilisation which would further decrease the NRR times. Based on this premise, we examined the effects of exposure to different salinities using the NRR assay, and comparing the impact of using different PSS.

A significant difference ($P < 0.001$) was found when the effects of using PSS 1 (38.5‰) and PSS 2 (32.5‰) were compared in clams that had been exposed to seawater of 35‰ (Fig. 1). When PSS 2 was used, few intact cells were found and a large amount of cell debris was seen. This was likely to have been a result of hypotonic stress exerted on the cells, which would ultimately lead to migration of water into the cells causing them to swell and burst. This illustrated the negative impact of using a PSS which had a lower salinity than that of the haemolymph. It is for this reason that the PSS used in this study were approximately 0.5–1‰ above that of the haemolymph. On further investigation following exposure to different salinities, significant differences ($P < 0.001$) were recorded between use of PSS that were adapted to more closely 'match' the salinity of the haemolymph, and when PSS 1 was used throughout the study (Fig. 1). This emphasizes the need to measure the salinity of the aquatic environment the organisms are inhabiting and their corresponding haemolytic salinity (as this may vary between species), and ascertain which PSS would be most appropriate in reducing adding additional osmotic stress. However, at the different salinities, a decline in the NRR times was also recorded when the PSS that were proposed to 'match' the haemolymph salinity were utilised, with a significant difference ($P < 0.05$) recorded between clams exposed to seawater salinity 23‰ and the other salinities (Fig. 1). This suggests that the adapted PSS were still inflicting some stress on the cells, and further work is needed to establish a suite of PSS that can be used at different salinities without causing additional hypo/hyper-osmotic stress.

Nicholson (2001) reported that hyposalinity can induce lysosomal membrane destabilisation as assessed by the NRR assay. However, throughout their study, they used the same PSS when measuring the effects of exposure to seawater of different salinities. We speculate that this could have added additional osmotic stress influencing the results they report. Dailianis et al. (2003) reported reduced NRR times below 50 min in all sampling stations, including the reference site. They suggested that the chemical composition of the PSS they used in the NRR assay could have had an impact on the low NRR times recorded. In a study by Marchi et al. (2004) using neutral red staining, the concentration of $MgSO_4$ in the PSS used was half than that used by Dailianis et al. (2003), and had a salinity of 38‰ (Table 1). Many marine invertebrates that are osmoconformers regulate the concentration of magnesium in their body fluids and keep this ion at a very low concentration (Schmidt-Nielsen, 1997). It is possible that the concentration of $MgSO_4$ in combination with a high concentration of NaCl used in the study by Dailianis et al. (2003), was too high and could have been the cause of extra adverse side effects being exerted on the lysosomal membranes stability. It might be more beneficial to keep the concentration of $MgSO_4$ low in line with that used by Marchi et al. (2004), but only change the concentration of NaCl to reach the required salinity and hence establish a uniform NRR assay that can be used at different salinities.

5. Conclusion

This study further validates the NRR assay as a powerful and sensitive biomarker of stressors, including those found naturally occurring in estuarine environments such as salinity fluctuations. In addition, we showed the negative effects of using PSS which featured salinities that imposed additional osmotic stress upon the haemolymph cells. Furthermore, we established the need to develop a suite of PSS that would not inflict hypo/hyper-osmotic stress on cells when haemolymph is collected. However, the adapted PSS used in this study were not ideal, as reductions in the NRR times were recorded at the reduced salinities, and further investigations are required to optimise PSS to be used at different salinities. This could improve and enhance the NRR assay by adding an extra element of conformity, making comparisons between studies from different salinities more accurate, and augment its capability as a quick, reliable and sensitive biomarker for future studies.

Acknowledgements

This research was funded by The Higher Education Authority under the Program of Research in Third Level Institutions (Cycle 2) as part of the Environmental Research Institute, University College Cork. We gratefully acknowledge the ongoing support of Ben Coughlan by the Disability Support Service at University College Cork.

References

Bayne, B.L., Addison, R.F., Capuzzo, J.M., Clarke, K.R., Gay, J.J., Moore, M.N., Warwick, R.M., 1988. An overview of the GEEP workshop. Mar. Ecol. Prog. Ser. 46, 235–243.

Blasco, J., Puppo, J., 1999. Effect of heavy metals (Cu, Cd and Pb) on aspartate and alanine aminotransferase in *Ruditapes philippinarum* (Mollusca: Bivalvia). Comp. Biochem. Physiol. C – Pharmacol. Toxicol. Endocrinol. 122, 253–263.

Brown, A.D., Simpson, J.R., 1972. Water relations of sugar-tolerant yeasts: the role of intracellular polyols. J. Gen. Microbiol. 72, 589–591.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H., 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267–278.

Byrne, P.A., O'Halloran, J., 2000. Acute and sublethal toxicity of estuarine sediments to the manila clam, *Tapes semidecussatus*. Environ. Toxicol. 15, 456–468.

Byrne, P.A., O'Halloran, J., 2001. The role of bivalve molluscs as tools in estuarine sediment toxicity testing: a review. Hydrobiologia 465, 209–217.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C., Viarengo, A., 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Tot. Environ. 247, 295–311.

Cheung, V.V., Wedderburn, R.J., Depledge, M.H., 1998. Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong. Mar. Environ. Res. 46, 237–241.

Coughlan, B.M., Hartl, M.G.J., O'Reilly, S.J., Sheehan, D., Morthersill, C., van Pelt, F., O'Halloran, J., O'Brien, N.M., 2002. Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments. Mar. Poll. Bull. 44, 1359–1365.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M., Dimitriadis, V.K., 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Mar. Environ. Res. 56, 443–470.

Depledge, M.H., 1993. The rational basis for the use of biomarkers as ecotoxicological tools. In: Fossi, M.C., Leonzio, C. (Eds.), Nondestructive Biomarkers in Vertebrates. Lewis Publishers, Boca Raton, FL, USA, pp. 261–285.

Depledge, M.H., Aagaard, A., Gyorkos, P., 1995. Assessment of trace metal toxicity using molecular, physiological and behavioural biomarkers. Mar. Biol. Bull. 31, 19–27.

Duquesne, S., Liess, M., Bird, D.J., 2004. Sub-lethal effects of metal exposure: physiological and behavioural responses of the estuarine bivalve *Macoma balthica*. Mar. Environ. Res. 58, 245–250.

Elston, R.E., Cheney, D.P., MacDonald, B.F., Suhrbier, A.D., 2001. Manila Clam Mortality and Health Evaluation. Pacific Shellfish Institute, Olympia, Washington. p. 1–23.

Elston, R.A., Cheney, D.P., MacDonald, B.F., Suhrbier, A.D., 2003. Tolerance and response of Manila clams, *Venerupis philippinarum* (A. Adams and Reeve, 1850) to low salinity. J. Shellfish Res. 22, 667–674.

Fernley, P.W., Moore, M.N., Lowe, D.M., Donkin, P., Evans, S., 2000. Impact of the *Sea Empress* oil spill on lysosomal stability in mussel blood cells. Mar. Environ. Res. 50, 451–455.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B., Depledge, M.H., 2004. A multibiomarker approach to environmental assessment. Environ. Sci. Technol. 38, 1723–1731.

Gosling, E.M., Nolan, A., 1989. Triploidy induction by thermal-shock in the Manila clam, *Tapes Semidecussatus*. Aquaculture 78, 223–228.

Hartl, M.G.J., Kilemade, M., Coughlan, B.M., O'Halloran, J., van Pelt, F., Sheehan, D., Mothersill, C., O'Brien, N.M., 2006. A two-species biomarker model for the assessment of sediment toxicity in the marine and estuarine environment using the comet assay. J. Environ. Sci. Health Part A – Toxic/Hazard. Subst. Environ. Eng. 41, 939–953.

Hideo, S., Ryo, I., 2003. Drastic decreasing of annual catch yields of the Manila clam *Ruditapes philippinarum* in Ariake sound, southern Japan. Umi no Kenkyu 12, 21–36.

ICES, WGBEC, 2004. Report of the Working Group on Biological Effects of Contaminants. Ostend, Belgium. p. 86.

Iversen, E.S., Hale, K.K., 1992. Aquaculture Sourcebook: A Guide to North American Species. Van Nostrand Reinhold, New York. p. 308.

Jensen, A.C., Humphreys, J., Caldow, R.W.G., Grisley, C., Dyrynda, P.J., 2004. Naturalization of the Manila clam (*Tapes philippinarum*), an alien species, and establishment of a clam fishery within Poole Harbour. Dorset. J. Mar. Biol. Ass. UK 84, 1069–1073.

Kim, W.S., Huh, H.T., Huh, S.H., Lee, T.W., 2001. Effects of salinity on endogenous rhythm of the Manila clam, *Ruditapes philippinarum* (Bivalvia: Veneridae). Mar. Biol. 138, 157–162.

Lam, P.K.S., Wu, R.S.S., 2003. Use of Biomarkers in Environmental Monitoring. City University of Hong Kong, Hong Kong. p. 5–6.

Livingstone, D.R., Chipman, J.K., Lowe, D.M., Minier, C., Mitchelmore, C.L., Moore, M.N., Peters, L.D., Pipe, R.K., 2000. Development of biomarkers to detect the effects of organic pollution on aquatic invertebrates: recent molecular, genotoxic, cellular and immunological studies on the common mussel (*Mytilus edulis* L.) and other mytilids. Int. J. Environ. Pollut. 13, 56–91.

Lowe, D.M., Fossato, V.U., 2000. The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels (*Mytilus galloprovincialis*) from the Venice Lagoon. Aquat. Toxicol. 48, 75–85.

Lowe, D.M., Soverchia, C., Moore, M.N., 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat. Toxicol. 33, 105–112.

Maddox, J., 1998. What Remains to be Discovered. Free Press, New York, p. 434.

Marchi, B., Burlando, B., Moore, M.N., Viarengo, A., 2004. Mercury- and copper-induced lysosomal membrane destabilisation depends on $[Ca^{2+}]_i$ dependent phospholipase A2 activation. Aquat. Toxicol. 66, 197–204.

Martins, L.K.P., Nascimento, I.A., Fillmann, G., King, R., Evangelista, A.J.A., Readman, J.W., Depledge, M.H., 2005. Lysosomal responses as a diagnostic tool for the detection of chronic petroleum pollution at Todos os Santos Bay. Brazil. Environ. Res. 99, 387–396.

McCarthy, J.F., Shugart, L.R., 1990. Biological markers of environmental contamination. In: McCarthy, J.F., Shugart, L.R. (Eds.), Biomarkers of Environmental Contamination. Lewis Publishers, Boca Raton, FL, pp. 3–14.

Melia, P., Gatto, M., 2005. A stochastic bioeconomic model for the management of clam farming. Ecol. Model. 184, 163–174.

Moore, M.N., 1985. Cellular responses to pollutants. Mar. Poll. Bull. 16, 134–139.

Moore, M.N., 2002. Biocomplexity: the post-genome challenge in ecotoxicology. Aquat. Toxicol. 59, 1–15.

Moore, M.N., Wedderburn, R.J., Lowe, D.M., Depledge, M.H., 1996. Lysosomal reaction to xenobiotics in mussel hemocytes using BODIPY-FL-verapamil. Mar. Environ. Res. 42, 99–105.

Moore, M.N., Allen, J.I., McVeigh, A., 2006a. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. 61, 278–304.

Moore, M.N., Allen, J.I., McVeigh, A., Shaw, J., 2006b. Lysosomal and autophagic reactions as predictive indicators of environmental impact in aquatic animals. Autophagy 2, 217–220.

Nasci, C., Da Ros, L., Campesan, G., Van Vleet, E.S., Salizzato, M., Sperni, L., Pavoni, B., 1999. Clam transplantation and stress-related biomarkers as useful tools for assessing water quality in coastal environments. Mar. Poll. Bull. 39, 255–260.

Nicholson, S., 1999a. Cardiac and lysosomal responses to periodic copper in the mussel *Perna viridis* (Bivalvia: Mytilidae). Mar. Biol. Bull. 38, 1157–1162.

Nicholson, S., 1999b. Cytological and physiological biomarker responses from green mussels, *Perna viridis* (L.) transplanted to contaminated sites in Hong Kong coastal waters. Mar. Poll. Bull. 39, 261–268.

Nicholson, S., 2001. Ecocytological and toxicological responses to copper in *Perna viridis* (L.) (Bivalvia: Mytilidae) haemocyte lysosomal membranes. Chemosphere 45, 399–407.

Nicholson, S., Lam, P.K.S., 2005. Pollution monitoring in Southeast Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). Environ. Int. 31, 121–132.

Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F., Frenzilli, G., 2006. Cellular biomarkers for monitoring estuarine environments: transplanted versus native mussels. Aquat. Toxicol. 77, 339–347.

Peek, K., Gabbott, P.A., 1989. Adipogranular cells from the mantle tissue of *Mytilus edulis* L. I. Isolation, purification and biochemical characteristics of dispersed cells. J. Exp. Mar. Biol. Ecol. 126, 203–216.

Regoli, F., 1992. Lysosomal responses as a sensitive stress index in biomonitoring heavy-metal pollution. Mar. Biol. Prog. Ser. 84, 63–69.

Ringwood, A.H., Conners, D.E., Hoguet, J., 1998. Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. Mar. Ecol. Prog. Ser. 166, 163–171.

Schmidt-Nielsen, K., 1997. Animal Physiology: Adaptation and Environment. Cambridge University Press, Cambridge, England. p. 607.

Slater, T.F., 1979. Biochemical studies on liver injury. In: Slater, T.F. (Ed.), Biochemical Mechanisms of Liver Injury. Academic Press, London, pp. 1–44.

Soudant, P., Paillard, C., Choquet, G., Lambert, C., Reid, H.I., Marhic, A., Donaghy, L., Birkbeck, T.H., 2004. Impact of season and rearing site on the physiological and immunological parameters of the Manila clam *Venerupis* (=*Tapes*, =*Ruditapes*) *philippinarum*. Aquaculture 229, 401–418.

Tay, K.L., Teh, S.J., Doe, K., Lee, K., Jackman, P., 2003. Histopathologic and histochemical biomarker responses of Baltic clam, *Macoma balthica*, to contaminated Sydney Harbour sediment, Nova Scotia, Canada. Environ. Health Persp. 111, 273–280.

Wedderburn, J., McFadzen, I., Sanger, R.C., Beesley, A., Heath, C., Hornsby, M., Lowe, D., 2000. The field application of cellular and physiological biomarkers, in the mussel *Mytilus edulis*, in conjunction with early life stage bioassays and adult histopathology. Mar. Poll. Bull. 40, 257–267.

Werner, I., Teh, S.J., Datta, S., Lu, X., Young, T.M., 2004. Biomarker responses in *Macoma nasuta* (Bivalvia) exposed to sediments from northern San Francisco Bay. Mar. Environ. Res. 58, 299–304.

Yancey, P.H., 2001. Water stress, osmolytes and proteins. Am. Zool. 41, 699–709.

Yancey, P.H., 2005. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. J. Exp. Biol. 208, 2819–2830.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D., Somero, G.N., 1982. Living with water-stress – evolution of osmolyte systems. Science 217, 1214–1222.

Zhang, G.F., Yan, X.W., 2006. A new three-phase culture method for Manila clam, *Ruditapes philippinarum*, farming in northern China. Aquaculture 258, 452–461.