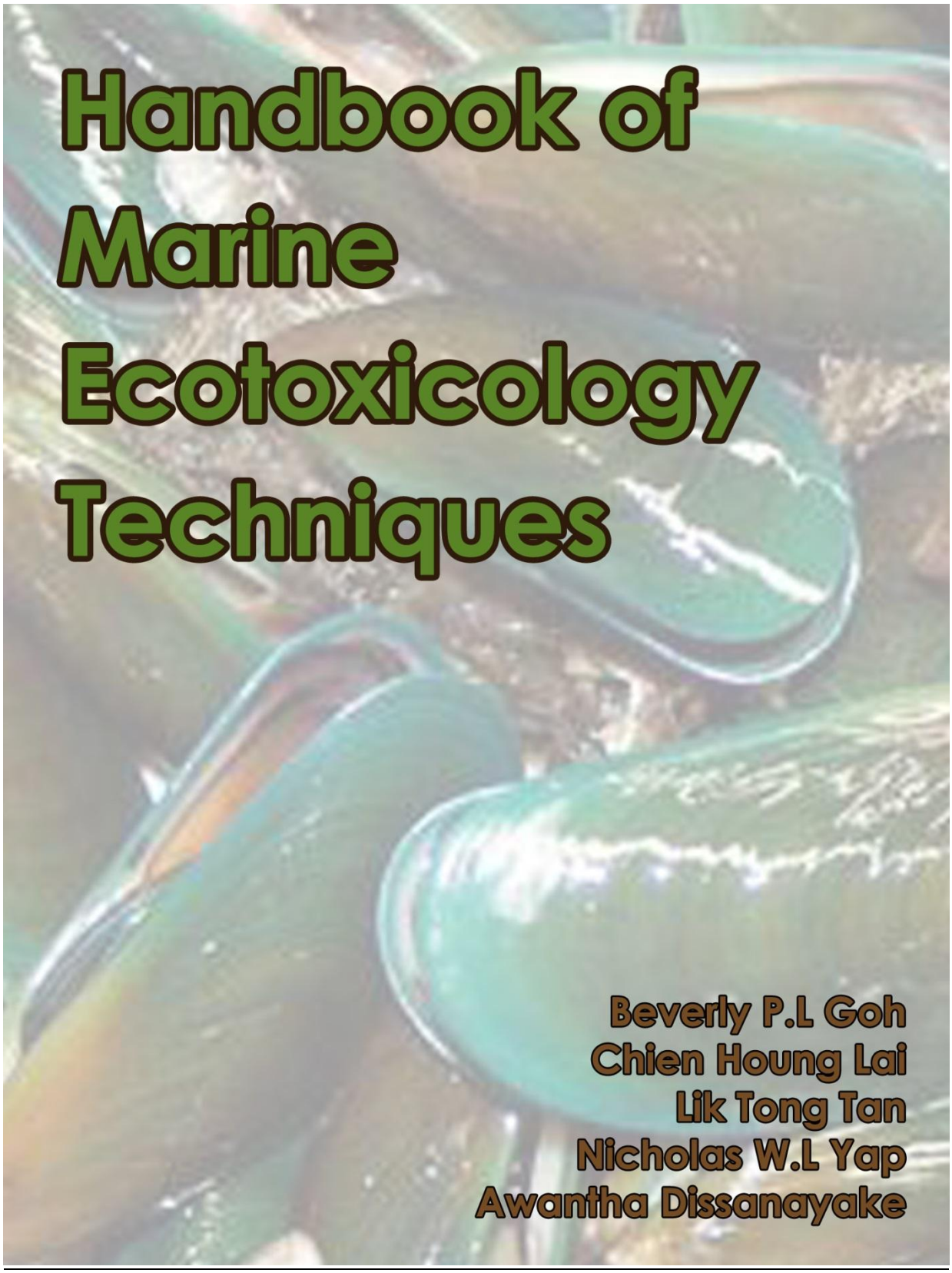
Handbook of
Marine
Ecotoxicology
Techniques
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Preface

Ecotoxicology is concerned with the effects of environmental toxicants on the health of organisms living in the natural environment. Environmental toxicants are agents released into the general environment that may cause adverse effects on health. The study of environmental toxicology stems from the recognition that:

(a) Human survival depends on the availability of clean air, water, and food and on the welfare of plants and animals and;
(b) Anthropogenic and naturally occurring chemicals can cause adverse effects on living organisms and ecological processes.

Environmental toxicology is a multidisciplinary science that encompasses several diverse areas of study that focuses on how environmental toxicants, through their interaction with humans, animals, and plants, influence the health and welfare of organisms. Related areas include biology; chemistry (inorganic, organic and analytical chemistry); anatomy; genetics; physiology; ecology; soil and water.

Over the decades, the concept of biomarkers has had a major impact in environmental sciences. The term is related to biological changes that can be observed in organisms under stress conditions that are either natural or environmental. The biomarker techniques covered in this handbook draws on different areas of biology: biochemistry, physiology, histology, and is studied at various organismal levels such as the molecular, cellular, physiological, organism, population, community or ecosystem.

This handbook emphasizes rapid, uncomplicated methods for assessing ecological, habitat, organismal and chemical integrity of marine habitats of Singapore. It provides fundamental information concerning the establishment of

survey techniques for chemical analysis of environmental toxicants, and biomarker assessment of fitness parameters from invertebrate organisms.

This book is intended to be of use to the general public, schools or policy makers, by providing a pragmatic approach to the use of biomarkers in the marine environment, and the implementation of a biomarker-based monitoring program. More generally, scientists in governmental agencies who have responsibilities at both the national and international levels will find a comprehensive list of techniques to set up a monitoring programme that will provide comparable chronological records of environmental health parameters.

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1 May 2014

CONTENTS

Anthropogenic Contaminants

Xenobiotic contaminants are very much varied and profound in the natural environment, and it would be impossible to exhaustively list either the possible occurring stressors themselves or their analytical procedures for detection. Broad categorizations of anthropogenic types with some common examples are illustrated here in Table 1.

Table 1. Sources of contaminants that are known to have negative impacts on the health of marine organisms and humans.

Heavy metals	· Cadmium (Cd) · Copper (Cu) · Zinc (Zn) · Lead (Pb) · Mercury (Hg)
Biocides and their by-products	· Aldrin · Dieldrin
Industrial organic chemicals	· Polychlorinated Biphenyls (PCBs) · Tetrachlorobenzene ($C_6H_2Cl_4$)
By-products of industrial processes and combustion	· Polycyclic Aromatic Hydrocarbons (PAHs) · Dioxins ($C_4H_4O_2$)

Heavy metals

The metals found in our environment are derived from a variety of sources, such as the natural weathering processes of Earth's crust, mining, soil erosion,

industrial discharge, urban runoff, sewage effluents, air pollution fallout, and a number of other sources.

While some metals found in our environment are essential nutritionally, others, like “heavy metals” are not. Heavy metals are a group of metallic elements that exhibit certain chemical and electrical properties and are generally those having a density greater than 5g/ml. These metals exceed the atomic mass of Calcium, and most are extremely toxic because of their solubility in ions or in compound forms. This translates to ready absorption into plant and animal tissue, and subsequently binding to biomolecules such as proteins and nucleic acids, impairing their functions.

Using Cadmium (Cd) as an example of heavy metal toxicity, the environmental hazard of this heavy metal first came into the spotlight with the historical outbreak of *itai-itai-byo* or “ouch-ouch disease” in Japan in 1945. The increased use of Cd and emissions from its production, as well as Lead and steel production, burning of fossil fuel, use of phosphate fertilizers, and waste disposal in the past several decades, combined with the long-term persistence of Cd in the environment, have lead many researchers to consider Cd to be one of the most toxic trace elements in the environment. Like other heavy metals, Cd binds rapidly to extracellular and intracellular proteins, thus disrupting membrane and cell function.

Biocides and their by-products

Chlorinated hydrocarbons, also called organochlorines, were the first commercial organic insecticides to be developed. Aldrin, chlordane, Dichloro Diphenyl Trichloroethane (DDT), dieldrin, endrin, lindane, and heptachlor are some examples. Organochlorine pesticides (OCP) such as aldrin and dieldrin are used as agrochemicals extensively until the 1970s. Nowadays, although OCPs have

been largely replaced by other kinds of pesticides (e.g. carbamates, synthetic pyrethoids and organophosphates) these chemicals are of major environmental concern as they are persistent organic pollutants (POPs, organic compounds that are resistant to environmental degradation through chemical, biological, and photolytic processes).

Industrial organic chemicals

Polychlorinated biphenyls (PCBs) are a class of synthetic chlorinated organic compounds with biphenyl as the basic structural unit. Although structurally belonging to chlorinated hydrocarbons, they are not pesticides. However, because of their wide use and resistance to degradation in the environment, PCBs are known as one of the major organochlorine pollutants found in the environment. Extensive PCB contamination exists in the food chain throughout the world.

Due to their toxicity and classification as a persistent organic pollutant, PCB production was banned by the United States Congress in 1979 and by the Stockholm Convention on Persistent Organic Pollutants in 2001. Concerns about the toxicity of PCBs are largely based on compounds within this group that share a structural similarity and toxic mode of action with dioxin. Toxic effects such as endocrine disruption and neurotoxicity are also associated with other compounds within the group. Therefore, the current maximum containment level as stated by the EPA for PCBs in drinking water systems is 0.5 ppb (parts per billion, or 0.5µg/l).

PCBs are very stable compounds and do not decompose readily. This is due to their chemical inability to oxidize and reduce in the natural environment. Furthermore, PCBs have a long half-life (8 to 10 years) and are insoluble in water, which contributes to their stability. Their destruction by chemical, thermal, and

biochemical processes is extremely difficult, and presents the risk of generating extremely toxic dibenzodioxins and dibenzofurans through partial oxidation.

By-products of industrial processes and combustion

Polycyclic aromatic hydrocarbons (PAHs), also known as poly-aromatic hydrocarbons or polynuclear aromatic hydrocarbons, are potent atmospheric pollutants that consist of fused aromatic rings and do not contain heteroatoms or carry substituents. Naphthalene is the simplest example of a PAH. PAHs occur in oil, coal, and tar deposits, and are produced as byproducts of fuel burning (whether fossil fuel or biomass). As a pollutant, they are of concern because some compounds have been identified as carcinogenic, mutagenic, and teratogenic.

Dioxins are by-products of various industrial processes, and are commonly regarded as highly toxic compounds that are environmental pollutants and persistent organic pollutants (POPs). The toxic effects of dioxins are measured in fractional equivalencies of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), the most toxic and best studied member of its class. Toxicity is mediated through interaction with a specific intracellular protein, the aryl hydrocarbon (AH) receptor, a transcriptional enhancer, affecting a number of other regulatory proteins. This receptor is a transcription factor which is involved in expression of many genes. TCDD binding to the AH receptor induces the cytochrome P450 1A class of enzymes which function to break down toxic compounds, e.g., carcinogenic polycyclic hydrocarbons such as benzo(a)pyrene. While the affinity of dioxins and related industrial toxicants to this receptor may not fully explain all their toxic effects including immunotoxicity, endocrine effects and tumor promotion, toxic responses appear to be typically dose-dependent within certain concentration ranges.

Chemical Analysis

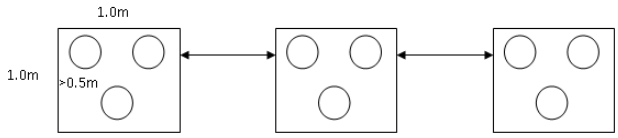
While analytical chemistry of environmental toxicants are often tedious, expensive, and rely on the use of very specialized analytical equipment, a yearly or quarterly sampling for major contaminants is usually recommended to be conducted routinely for background analysis. Routine monitoring is particularly important in environments facing risk from anthropogenic incidents like oil spills or chemical leakage from off-shore industries. These risks are constant challenges in the coastal region of Singapore.

As chemical analytical methods and equipment used in the final analysis of contaminants have their own specific technical instructions and optimization techniques, only fundamental sample collection and processing techniques are briefly covered in this chapter.

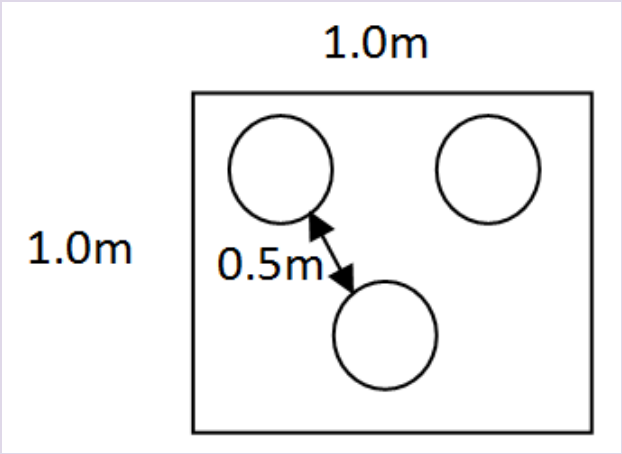
Typically, site collections can be divided into sediment, water and biota samples.

Sediment collection

Sediment collection can be performed as illustrated below:



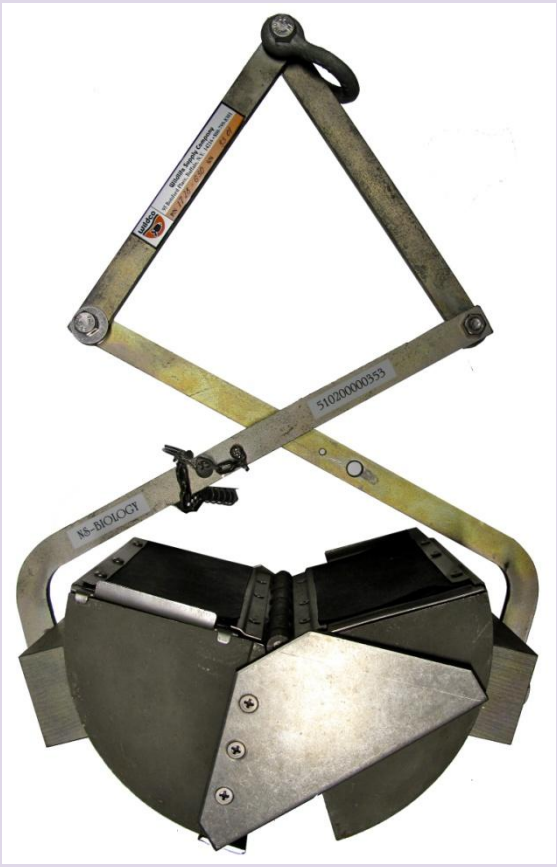
At each site, collect sediment samples at three separate points. Each point covers an area of 1m x 1m.



Within each point, collect sediment from three spots (diameter approximately 10cm) which are at least 0.5m apart from each other and homogenize the sediment in a tray:

- Transfer 100mL of homogenized sediment sample into a labeled glass jar (processed for trace analysis). Example of labelling format: *date-sites-sample number* 14Nov13-Changi-1.

- Collect at least three sediment samples from each site.



A bottom grab sampler

- If bottom sediment is to be sampled, a *Peterson* or *Van Veen* grab can be deployed.
- Transport the samples to the laboratory in a cool box.
- Record pH of the sediment.
- Air-dry the samples (40 – 50°C).
- Remove stones, shells and other oversized materials; and then sieve/ grind the sediment prior to further processing.

Water sample collection

Water sample collection can be performed using a horizontal water sampler as shown in the illustration below:

A horizontal water sampler

- Horizontal Water samplers are ideal for thin layer temperature and chemical stratification monitoring.
- It is important to move the sampler from side to side at the desired stratum to ensure sufficient "flow-thru" before closure.
- Record the time.
- Label the samples similar to sediment samples described above.
- Transport the samples to the lab in a cool box.
- Abiotic measurements like dissolved oxygen, temperature, pH and salinity should be conducted *in situ*.

Biota sample collection

Biota sampled could be either epifauna (organisms living on the surface of the seabed or attached to submerged objects) or infauna (organisms living in the sediments of the seabed). Consistency should be observed in selecting biota samples, e.g. choosing the same organisms/species between different sites for comparison, and noting the tidal height at which the animals were collected.

Typically, mussel species are be ideal candidates for analysis. Examples of mussel species that occur in the coastal waters of Singapore include *Perna viridis*, *Xenostrobus atratus*, *Mytilopsis sallei*, and *Musculista senhousia*. Depending on the sampling design, either the whole organism could be used in chemical analysis, or certain body parts could be targeted for analysis: shell, digestive glands, foot muscle, mantle, gills.

Marine Ecotoxicological Assays

Of central importance in both toxicology and ecotoxicology is the relationship between the quantity of chemical to which an organism is exposed and the nature and degree of consequent harmful (toxic) effects. Dose-response relationships provide the basis for assessing hazards and risks presented by environmental chemicals. However, it should be recognized that no chemical is poisonous if a dose is low enough. Conversely, all chemicals are poisonous if doses are high enough (e.g. salt and salinity).

Toxicity can be measured in a number of ways. Most commonly, the end point is death, although interest in more sophisticated, sub-lethal indices is growing. To that effect, biochemical, physiological, reproductive, and behavioural effects can provide sub-lethal measures of toxicity. More importantly, it has become ecologically relevant that population declines in wildlife species are the consequences of sub-lethal rather than lethal effects of pollutants. In these cases, fitness is compromised and biological functions are not fully functional despite organismal survival. For example, reproductive failure events will are not captured by lethal measurements of toxicity effects, and endocrine disruptors that affect communication ability, or disrupt foraging behaviour would similarly be overlooked by traditional toxicity lethal limits.

Many toxicity tests provide estimates of doses that will cause a toxic response at the 50% level, e.g. the median lethal dose that kills 50% of a population. For an introduction to the ecotoxicological tests in this handbook, several terms used in relation to toxicity testing require definition.

LD_{50}	Median lethal dose at which 50% of test organisms die
LC_{50}	Median lethal concentration at which 50% of test organisms die
NOED	No-observed-effect dose: highest dose that causes no toxicity
NOEC	No-observed-effect concentration: highest concentration that causes no toxicity
ED_{50}	Dose at which a non-lethal response is produced in 50% of the population
EC_{50}	Concentration at which a non-lethal response is produced in 50% of the population

In addition to toxicity tests on live animals covered in this handbook, other methods can evaluate the toxic properties of chemicals that stem from understanding the modes of action of chemicals. For example, bacterial mutagenicity assays (e.g. the Ames test) help identify substances that can act as carcinogens or mutagens in mammals.

As opposed to chemical analysis of contaminants in a marine habitats, ecotoxicology assays measure the parameters of fitness of an organism, and are thus integrated measurements of stress. Although the causative agent of the stress may not be immediately recognized (unless in contaminant-specific test, like metallothionein production with exposure to heavy metals, or genotoxic response to carcinogenic contaminants), ecotoxicological assays provide rapid, relatively simple and cheap analysis and assessments of organismal, community and habitat health and integrity. Resources may then be reserved for chemical analyses only in areas and habitats yielding less than desirable fitness test results.

It should be noted, however, that a single ecotoxicology assay on its own will not yield too much information on habitat or organismal health for two notable reasons:

(a) There is no index threshold at which a dichotomous healthy or unhealthy demarcation can be made: a background threshold for comparison must be established, and thus a routine monthly or quarterly survey conducted would greatly strengthen the results from the ecotoxicological test being employed.

(b) Just as contaminants could target different levels of biological functions or have varying degrees of stress effects, a suite of biomarker techniques targeted at behavioural, physiological, biochemical, and reproductive functions would be ideal to fully capture the effects of environmental stressors on test organisms.

Alternatively, controlled laboratory manipulation could also create a background threshold for field comparison. A total of 12 ecotoxicological assays (A – L) are presented in this handbook. Apart from the Sponge Aggregation assay which uses sponge cells, the other assays were developed specific to the local green lipped mussel *Perna viridis*. Tables 2 and 3 show EC_{50} values derived from the described techniques optimized in laboratory experiments, to provide rough estimations of toxicity dose responses with either anthropogenic contaminants or environmental stressors (Goh and Lai, 2013).

For the purpose of the handbook, only simple, rapid and relatively cheap methods are described, for synchronization of habitat monitoring by educational bodies or interest groups. This will facilitate a long-term monitoring programme of marine habitat health of the coastal waters of Singapore. Although *Perna viridis* is used in this book as a model organism to optimize most of the techniques, the

assays can be applied to a broad range of organisms with similar physiology and/or body plans, e.g. marine gastropods. As long as consistency of species choice is maintained, a meaningful stress index can be established over time.

Table 2. Sample of EC_{50} values obtained from specific biomarker techniques exploring the effects of heavy metals. Concentrations are in ppb.

Biomarker assays	Time of exposure	Copper	Cadmium
Clearance rate	24 h	787	79
	48 h	645	65
Neutral Red Retention Time	24 h	86	80
	48 h	78	65
Sponge aggregation	24 h	465	332
	48 h	600	286
Byssus Thread Count	24 h	399	
	48 h	504	
Fertilisation	1 h	150	
	3 h	1790	
	6 h	3560	

Table 3. LT_{50} /EC_{50} values obtained from specific biomarker techniques examining effects of pesticide exposure and heat stress (Lethal temperature or Effective Concentration).

Biomarker assays	Time of exposure	Stressor	LT_{50}/EC_{50}
Fertilization	24 h	Aldrin	1800ppb
Total Glutathione Assay	24 h	Dieldrin	2000ppb
	24 h	Cadmium	92.8ppb
	24 h	Heat stress	30.73ºC
Ferric Reducing Power Test	24 h	Heat stress	32.60ºC
Glycogen analysis	24 h	Heat stress	31.40ºC
Heat shock protein	24 h	Heat stress	32.10ºC

Test organisms

The ecotoxicology assays in this handbook were optimized using two sponge species (*Callyspongia* sp. and *Haliclona* sp.) and a local green-lipped mussel, *Perna viridis.* However, other marine species abundantly found in the coastal waters of Singapore may be employed for the techniques, after some further optimization and trial testing. In this handbook, a general guide to sponge and mussel biology and anatomy is illustrated.

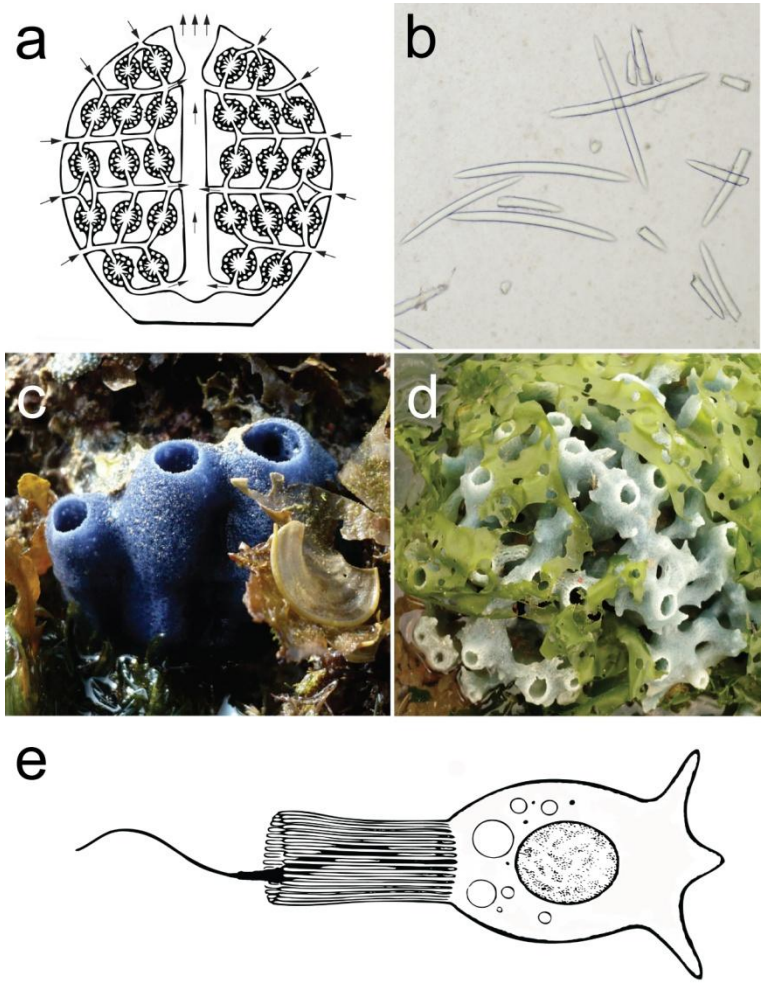
Kingdom *ANIMALIA*

Phylum *PORIFERA*

Classes

CALCAREA, DEMOSPONGIAE,

SCLEROSPONGIAE, HEXACTINELLIDA



Above. Sponges. a) Cross-section of a typical sponge. Arrows denote water flow, adapted from Pearse and Buchsbaum (1987); b) Sclerites of a sponge; c) and d) Some sponges found in Singapore's marine habitats; e) Choanocyte of a sponge, adapted from Pearse and Buchsbaum (1987)

Sponges (Phylum Porifera) are very simple animals that live permanently attached to a substrate in the water - they are sessile as adults. There are about 5,000 to 10,000 known species of sponges, with about 7000 species described to date (Hooper & Soest 2002). Most sponges live in salt water - only about 150 species live in fresh water. Sponges evolved over 500 million years ago. They are among the oldest of the multicellular animal (Metazoa), and possess relatively little in the way of differentiation and coordination of tissues (Bergquist 1978; Simpson 1984).

Marine sponges represent a significant component of benthic communities throughout the world, in terms of both biomass and their potential to influence benthic or pelagic processes (Dayton 1989; Gili & Coma 1998). They are sessile, filter-feeding organisms which, despite a simple body plan, are remarkably efficient at obtaining food from the surrounding water (Reiswig 1971; Vogal 1977).

The body of this primitive animal has thousands of pores which allow water to flow through it continually. Sponges obtain nourishment and oxygen from this flowing water. The flowing water also removes waste products.

Anatomy – The body of a sponge has two outer layers separated by an acellular (having no cells) gel layer called the mesohyl (also called the mesenchyme). In the gel layer are either spicules (supportive needles made of calcium carbonate) or spongin fibers (a flexible skeletal material made from protein). Sponges have neither tissues nor organs. Different sponges form different shapes, including tubes, fans, cups, cones, blobs, barrels, and crusts. These invertebrates range in size from a few millimeters to 2 meters in height.

Diet **–** Sponges are filter feeders. Most sponges eat tiny, floating organic particles and plankton that are filtered from the water that flows through their bodies. Food is collected by specialized cells called choanocytes (collar cells) and brought to other cells by amoebocytes.

Reproduction – Most sponges are hermaphrodites (each adult can act as either the female or the male in reproduction). Fertilization is internal in most species; some released sperm randomly float to another sponge with the water current. If a sperm is caught by another sponge's choanocytes, fertilization of an egg by the traveling sperm takes place inside the sponge. The resulting tiny larva is released and is free-swimming; it uses tiny cilia (hairs) to propel itself through the water. The larva eventually settles on the sea floor, becomes sessile and grows into an adult.

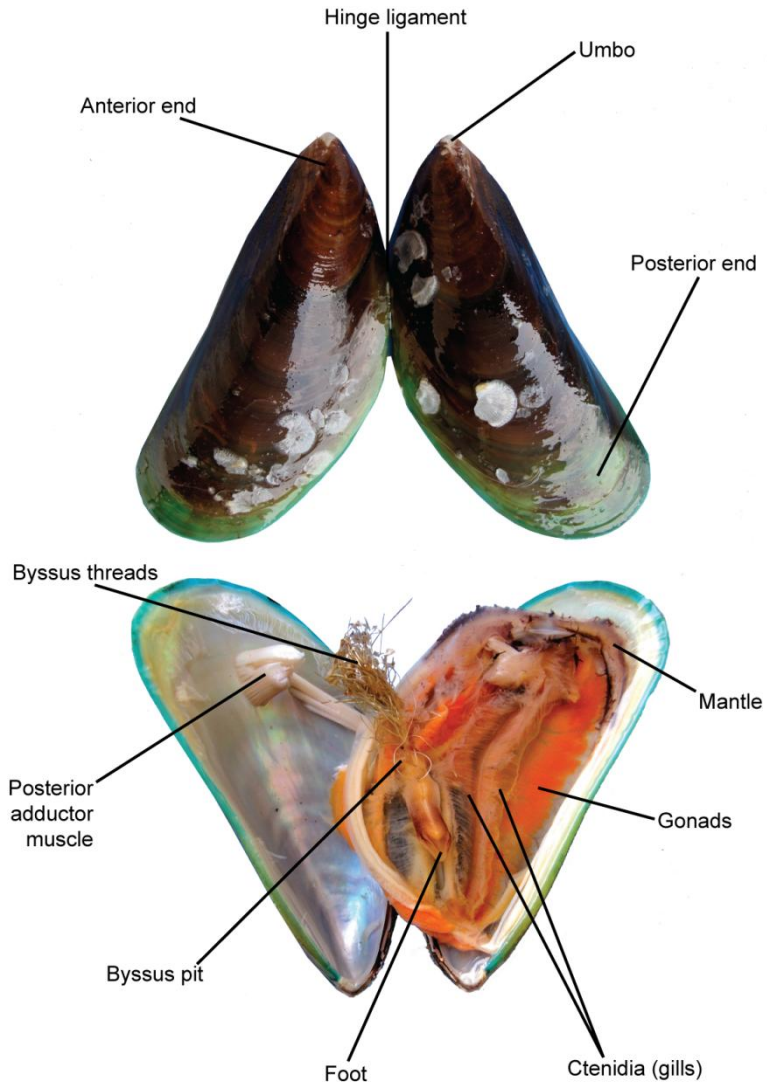
Some sponges also reproduce asexually; fragments of their body (buds) are broken off by water currents and carried to another location, where the sponge will grow into a clone of the parent sponge. i.e., its DNA is identical to the parent's DNA.

Kingdom *ANIMALIA*

Phylum *MOLLUSCA*

Class *BIVALVIA*

Subclass *PTERIOMORPHA*



Above: Anatomy of *Pernas viridis*. *Top*: the external shell morphology; *below*: internal morphology of *P. viridis.*

Although the green-lipped mussel *Perna viridis* is used as the test organism in this handbook, the techniques can be employed on any mussel species. As such, a basic mussel anatomy is illustrated here.

External anatomy – The mussel's external shell is composed of two valves that protect it from predators and desiccation.

Foot – Like most bivalves, mussels have a large organ referred to as a foot, which is tongue-like in shape with a groove on the ventral surface, which is continuous with the byssus pit. In this pit a viscous secretion is poured out which enters the groove and hardens gradually when it comes into contact with sea water. This forms an extremely tough byssus thread that secures the mussel to its substrate.

Feeding – Both marine and freshwater mussels are filter feeders that feed on plankton. They do so by drawing water in through their incurrent siphon. The water is then brought into the branchial chamber by the actions of the cilia located on the gills for cilliary-mucus feeding. The waste water exits through the exhalent current siphon. The labial palps finally sort the particles the food is funneled into the mouth where digestion takes place.

Clumping – Marine mussels are usually found clumped together on wave-washed rocks. Their byssus threads and the clumping behaviour anchors them onto the hard substrate and provides protection against the force of waves. Mussels found in the middle of a clump will experience less water loss due to water capture by the other mussels.

A. Sponge aggregation assay

Research on marine sponges has indicated that they have potential to be used as bioindicators of heavy metal pollution (Vogel 1977). Sponges prove to be potentially better bioindicators compared to other filter-feeders like bivalves and tunicates because they possess no internal organs and are specifically designed for filtering large volumes of water, between 100 and 1200 ml h-1 g-1 sponge (Vogel 1977; Riisgard et al. 1993). In addition, sponges are also unselective filter-feeders that can retain particles, mainly bacteria and ultraplankton, up to 8 µm diameter (Barthel & Wolfrath 1989).

Benthic marine sponges inhibiting shallow waters are tolerant to small amounts of copper but are susceptible at levels above threshold concentrations, and display toxic effects (Peña et al. 1999). It has been established that high levels of copper in waters have a negative effect on marine invertebrates such as bivalves and sponges (Reish et al. 1984). Industrialisation has increased the level of heavy metal pollution in recent years, e.g. copper, through discharge of wastes into rivers (Gladstone & Dight 1994; Gladstone 1996). The increased use of copper-based antifouling paint in the shipping industry has also introduced additional sources to copper in the marine environment (Claisse & Alzieu 1993). Exposure of marine organisms to elevated copper from anthropogenic sources thus warrants a need for monitoring the pollution levels.

Studies have shown that copper, zinc and cadmium accumulated by the sponge, *Halichondria panicea*, are positively correlated with concentrations in the surrounding media (Olesen & Weeks 1994; Hansen et al. 1995). High metal accumulation rates were measured with no apparent loss of accumulated metals in the sponge even after transfer to clean seawater for flushing of the canals within the sponge. Dissociated sponge cells are known to form functional aggregates. Sponge tissue placed in artificial sea water free of calcium and magnesium result

in sponge cells being dissociated from the silica matrix to exist as individual cells in a suspension. Subsequent calcium added to the suspension induces the cells to secrete a species-specific proteoglycan aggregating factor, causing cells to re-aggregate (Galtsoff 1925). They first form small primary aggregates of only a few cells before forming secondary aggregates. The secondary aggregates then rearrange themselves to form re-aggregated sponges that have water-channels similar to the original sponge (Müller 1977). In view of the large volumes of water passing through the bodies of sponges, slow depuration of heavy metals and sponges possessing the natural unique ability to re-aggregate, they are suitable candidates for water toxicity studies, and as bioindicators of heavy metal pollutants (Hansen et al. 1995; Webster et al. 2001; Goh 2008).

Chemicals and Equipment

- ☑ Haemocytometer
- ☑ 100 ml acid-washed glass beakers
- ☑ Siliconized eppendorf tubes (1.5, 2ml)
- ☑ Micropipette (1ml, 100µl)
- ☑ Compound microscope
- ☑ Calcium chloride (Sigma, #C1016-100G)
- ☑ Calcium-Magnesium Free Sea Water (CMFSW)

Preparation

Calcium-Magnesium Free Sea Water (CMFSW; Philp, 1997)

Reagent	Final concentration (mM)
NaCl	460
KCl	10
Na_2SO_4	7
HEPES buffer (pH 7)	10
2Na-EDTA	2.5

Method

1. Hand-collect a single, large piece of sponge specimen from sampling location where the specimen grows naturally.

2. Record the GPS location of the sampling site.

3. Use the entire piece of large sponge in the experiment to ensure the reduction of possible genetic variability.

4. Transport the sponge (stored in aerated seawater) to the laboratory (within 30 minutes).

5. Record the following information on sample collection day:
   · Salinity · pH (seawater at sampling site).

6. Sufficiently clean the sponge in filtered artificial seawater (ASW) (using Red Sea Salt; 0.45µm, salinity 29‰) to remove any associated symbionts (e.g. synaptids).

7. Briefly cut and rinse the sponge (approximately 1g) for 5mins, in calcium, magnesium-free seawater (CMFSW, pH 7).

8. To obtain the required cell suspension, cut the sponge into fine pieces, followed by rinsing (for 15mins) with fresh CMFSW.

9. Pipette 1.5ml of the resultant cell suspension into a 1.5ml siliconised centrifuge tube and homogenize with a vortex mixer.

10. Place 0.5ml of the homogenised cell suspension in a haemocytometer.

11. Count the number of cells and aggregations (3 or more cells joined together) in the mixture under a compound microscope.

12. To induce cell aggregation, add 50µl of 24mM $CaCl_2$ to the remaining 1ml of homogenised cell suspension.

13. Agitate the centrifuge tube for a period of 15min (using a vortex mixer) prior to re-counting the numbers of single and aggregated cells using the haemocytometer.

14. Express the mean proportion of single cells (after induced aggregation by $CaCl_2$) by dividing the number of single cells after induced aggregation by the number of single cells before induced aggregation.

BEHAVIOURAL / PHYSIOLOGICAL ASSAYS

B. Clearance rate (mussels/bivalves)

Being sessile filter feeders, water processing capabilities of *Perna viridis* directly translates to feeding efficacy and is integral to organismal health and survival. Studies on physiological responses of bivalves have shown reduction in feeding rates with exposure to environmental stress factors like sedimentation (Bricelj and Malouf, 1984; Ward and MacDonald, 1996; Bacon et al., 1998), hypoxia (Wang et al, 2011) or heavy metals (Watling, 1981; Grace & Grainey, 1987).

There is, however, great contention over the physiology of feeding rates of mussels in academic circles. While one view holds that feeding rate is actively regulated by the animal in response to environmental conditions (Bayne, 1993, 1998, 1999, 2000; Bayne, et al., 1993; Cranford, 2001; Cranford and Hill, 1999; Hawkins et al., 1999; Widdows, 2001), this postulation is challenged by another hypothesis that clearance is an autonomous process that is largely controlled by the physical properties of seawater, in particular viscosity (Jorgensen, 1990, 1996; Jorgensen et al., 1986, 1988, 1990; Riisgard, 2001a,b; Riisgard and Larsen, 2007; Riisgard et al., 2003).

Feeding activity, however, requires energy, fuelled by reserves (Kooijman, 2010), and is likely driven by biochemical processes and physiological competency (Chiu et al., 2007; Pechenik et al., 2002). It is also observed that mussels tend to close their valves and subsist on anaerobic respiration, i.e. no filtering, when exposed to heavy metals (Sloff et al., 1983). Thus, the water processing efficiency of mussels can still be used as a behavioural index to assess physiological stress in mussels.

Establishing dosage dependency of how this basic function is affected by environmental pollutants is a useful assessment tool in biomonitoring. The volume of seawater filtered by the mussels can be assessed by means of microalga feed removal (clearance rate) from a fixed volume of water (Coughlan, 1969).

Chemicals and Equipment

- ☑ Crystallization dishes (150mm x 75mm)
- ☑ Microalga feed (Shellfish Diet 1800®, Reed Mariculture)
- ☑ Haemocytometer
- ☑ Micropipette (capable of 20~200µl aliquots)
- ☑ Compound microscope

Method

1. Fill each crystallization dish with 500ml of artificial seawater.

2. Add 1ml of microalga feed to each dish.

3. Provide continuous aeration for each dish.

4. Stir the solution to ensure uniform mixing.

5. Aliquot 200µl of seawater and microalga feed mix from each dish into an eppendorf tube or beaker.

6. Enumerate the microalga cells (indicated by arrows in the figure) under a haemocytometer (20µl) and calculate the density of the cells.

7. Place 2 *Perna viridis* specimens from each treatment tank into the crystallization dish.

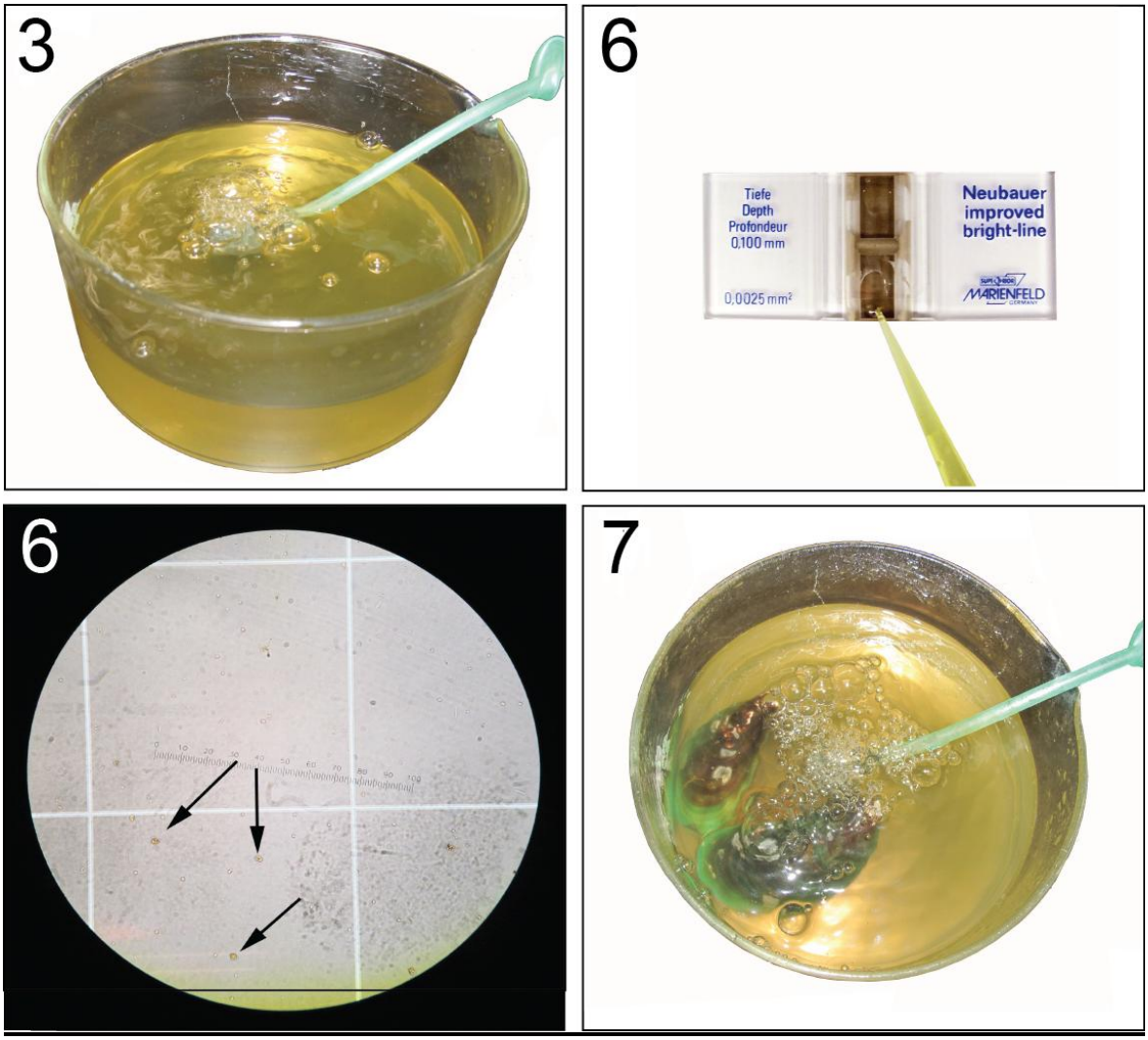
8. 5 replicates (N=10 mussels) should be used for each experimental condition (control inclusive).

9. After 1 hr repeat steps 4 to 6.

10. Clearance rate per test mussel (CR, Lh^{-1}) is calculated with the formula:

$$CR = \frac{60V\,[(\ln C_0 - \ln C_1)]}{nt1000}$$

where, V is the volume of the water in the crystallization dish (ml), C_0 the initial concentration of microalgae (cells/ml), C_1 the final concentration of microalgae , n the number of test organisms in each dish and t the time (h) between C_0 and C_1.



The above pictures illustrate procedures involved in steps 3, 6, and 7 on pages 26-27.

C. Byssus thread count

Byssus (or byssal) threads are proteineous materials secreted by the byssus gland at the base of the foot of mussels for anchorage on hard substratum (Yonge 1962; Price 1983), and as a predatory escape to immobilise predators (Farrell and Crowe 2007). The dynamic process of byssus secretion in mussels is influenced by a number of both exogenous and endogenous factors, like energy status of the individual (Clarke, 1999) or environmental factors (Zardi et al, 2007). Variations in byssus thread production with changes in environmental conditions have been reported for several species of mussels (Winkle, 1970; Price, 1980; Meadows and Shand, 1989), and this could potentially serve as a biomonitoring tool for environmental contamination.

It has been observed that detached mussels invariably try to reattach themselves (Clarke and McMahon, 1996) and in doing so, exhibit increased foot activity and byssus thread production (Young, 1985). Considering that byssus thread production, especially replacement of decayed byssus threads, could be highly demanding in energy expenditure (Griffliths and King, 1979; Hawkins and Bayne, 1985), physiological competence and individual energy reserves of the mussel might be limiting factors in byssus thread secretion under certain stressful conditions (Lachance et al, 2008). Conversely, the primary response of mussels to environmental stressors like heavy metal or salinity shock is to close their valves and subsist on anaerobic respiration (e.g. Lewis, 1985). When energy supply is limited by aerobic metabolism, byssus thread production has to compete with other physiological processes for energy. The balance of energy allocation to byssus thread production and physiological processes like tolerance to environmental contaminants is thus often a survival strategy of priority needs.

Chemicals and Equipment

- ☑ Crystallization dishes (150mm x 75mm)
- ☑ Glass Petri dish
- ☑ Scalpel/scissors

Method

1. Fill each crystallization dish with 800ml of the appropriate treatment solution (artificial seawater for control).

2. Provide continuous aeration for each dish.

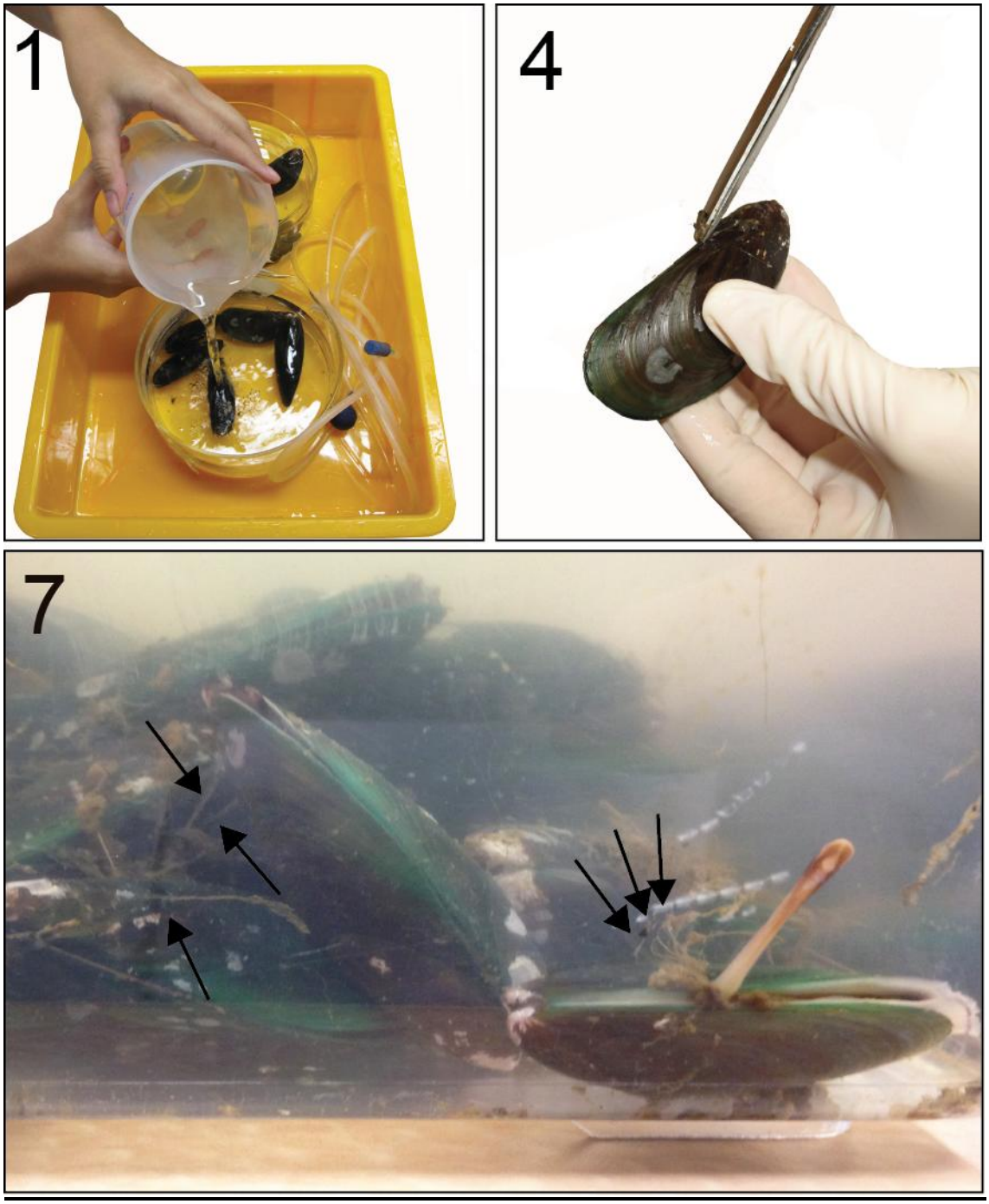
3. Cover each dish with a glass Petri dish to minimize evaporation.

4. Remove (cut) byssus threads protruding from the shell of the mussel as close as possible to the edge of the valves without damaging the byssus gland and pedal apparatus.

5. Place 2 mussels from each treatment tank into the appropriate dish with the dorsal side facing the side of the crystalline dish.

6. Five replicates (N=10 mussels) should be used for each experimental condition (control inclusive).

7. After 24 hours score the number of byssus threads produced by each mussel.



The above pictures illustrate procedures involved in steps 1, 4 and 7 on pages 30.

CELLULAR ASSAYS

D. Neutral Red Retention

Molluscs have organs and tissues which are highly dependent on an extensively developed lysosomal system: subcellular organelles surrounded by a semipermeable membrane that contain numerous hydrolytic enzymes involved in a range of cellular processes including digestion, defense, and reproduction (Moore, 1976; Pipe, 1993; Ferreira and Dolder, 2003). Lysosomes play an important role in the detoxification and defense in marine organisms (Moore, 1980; Lowe et al., 1995a, b), and may accumulate a variety of toxic metals and organic chemicals. Enhanced catabolic activity and lysosomal damage can provoke, as an ultimate effect, the leakage of acidic hydrolases in the cytosol, possibly leading to more severe damage and to cell death (Koehler et al., 2002). Recent findings suggest that toxic chemicals may affect these organelles not only directly on the membrane, but also by activating (or downregulating) calcium- and tyrosine kinase-dependent cell signaling pathways (Burlando et al., 2002; Canesi et al., 2004; Marchi et al., 2004). As these functions are a membrane-dependent process, the stability of the lysosomal membrane can 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 eurhodin dye 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 and the efficiency of its membrane-bound proton pump (Seglen, 1983) that maintains the acid condition within lysosomes (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 dye will

leak into the cytosol of the cell much more quickly (Moore, 1980; Lowe et al., 1992). The Neutral Red Retention assay is commonly used as a biomarker 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). See Luzio et al. (2007) and Moore et al. (2008) for a review on lysosomal characteristics and their physiological and immunological functions.

The measurement of lysosomal membrane integrity is presently considered a widely accepted index of environmental quality because they i) have been shown to be the target for a wide range of contaminants, ii) are easy to visualize in blood cells and in reacted tissue cryosections, and iii) are present in all nucleated cells and therefore are not species-specific.

Chemicals and Equipment

- ☑ Neutral Red (Sigma Aldrich #N4638)
- ☑ Hepes (Sigma Aldrich #90909C)
- ☑ Sodium chloride (Sigma Aldrich #S7653)
- ☑ Magnesium sulphate (Sigma Aldrich #M7506)
- ☑ Potassium chloride (Sigma Aldrich #P9333)
- ☑ Calcium chloride (Sigma Aldrich #C1016)
- ☑ Dimethyl sulfoxide (Sigma Aldrich #D9170)
- ☑ Poly-L-lysine 0.1 % (w/v) in H_2O (Sigma Aldrich #P8920)
- ☑ Siliconzied eppendorf tubes 1.7mL (Sigma Aldrich #T3406)
- ☑ Microscope glass slides & cover slips
- ☑ Microcentrifuge tubes 1.7 ml
- ☑ Phosphate Buffer Solution (pH 7.4)
- ☑ 21 G needles
- ☑ 1ml syringes
- ☑ Compound microscope
- ☑ Light proof humidity chamber
- ☑ Micropipette (40µl)

Preparation

i) Poly-L-Lysine Coating of the Microscope slides

Poly-L-Lysine is diluted with distilled water at a ratio of 1:10, e.g. 10µl Poly-L-Lysine in 100µl distilled water. Apply 10µl of the diluted solution to each clean microscope slide and smear it over the surface to the slide with a cover slip. Allow to air dry. Purpose: to make the slide surface sticky to cells.

ii) Marine Bivalve Physiological Saline

To be prepared in advance of blood sampling. Can be stored at 4°C for up to one month.

Hepes	4.77g
Sodium Chloride	25.48g
Magnesium sulphate	13.06g
Potassium chloride	0.75g
Calcium chloride	1.47g

Make up to 1 litre with distilled water, adjust pH to 7.36. Store in the refrigerator, but use at room temperature. This is to ensure that the cells are not stressed by temperature shock when extracted from the animals.

iii) Neutral Red (NR) Stain

Stock Solution: Can be prepared up to 2 weeks before the experiment.
Working Solution: Prepared immediately prior to use.

Stock Solution: 20mg of NR powder weighed into a smoked amber vial, and dissolved in 1ml dimethylsulfoxide (DMSO). Filter the solution through a 0.45µm filter. Store in the dark at 4°C.

Working Solution: Remove the stock solution from the fridge and allow it to reach room temperature (and thus become liquid). Transfer 995µl of physiological solution into a 2ml smoked vial, then add 5µl of the NR stock solution, mix by vortexing. Note: The neutral red solutions (both stock and working) should be maintained in the dark to prevent photo-oxidation wherever possible.

Method

1. Carefully open up the valves of the mussel using a pair of scissors, or a fixed scalpel blade, and allow excess seawater to drain out from the shell cavity.

2. Draw 0.5ml physiological saline into the syringe, then extract 0.5ml of haemolymph from the posterior adductor muscle, into the same syringe.

3. Remove the needle from the syringe and gently transfer the contents into a pre-siliconised microcentrifuge tube.

4. When all the mussels have been bled, transfer 40µl if the haemolymph mixture onto the centre of a Poly-L-Lysine coated microscope slide, using a fresh micropipette tip for each sample.

5. Immediately place the slide into a light-proof humidity chamber, and incubate for 15 minutes.

6. Following the incubation period, tap off the excess physiological saline/haemocyte mixture.

7. Then add 40µl of the working neutral red solution to the cells on the slide.

8. Replace the slides into the humidity chamber, and incubate for a further 15 minutes to allow the dye to penetrate the lysosomal compartment of the cells.

9. Minimally expose the treated cells to light due to the photosensitive nature of neutral red.

10. Place a coverslip on the slide avoiding the introduction of air bubbles.

11. Quickly and systematically examine each slide under the light microscope at 15mins intervals for the first hour followed by intervals of 30mins thereafter, at 40x magnification, refer to the figure on page 38 and steps 12 to 15 to determine the type of cells present.

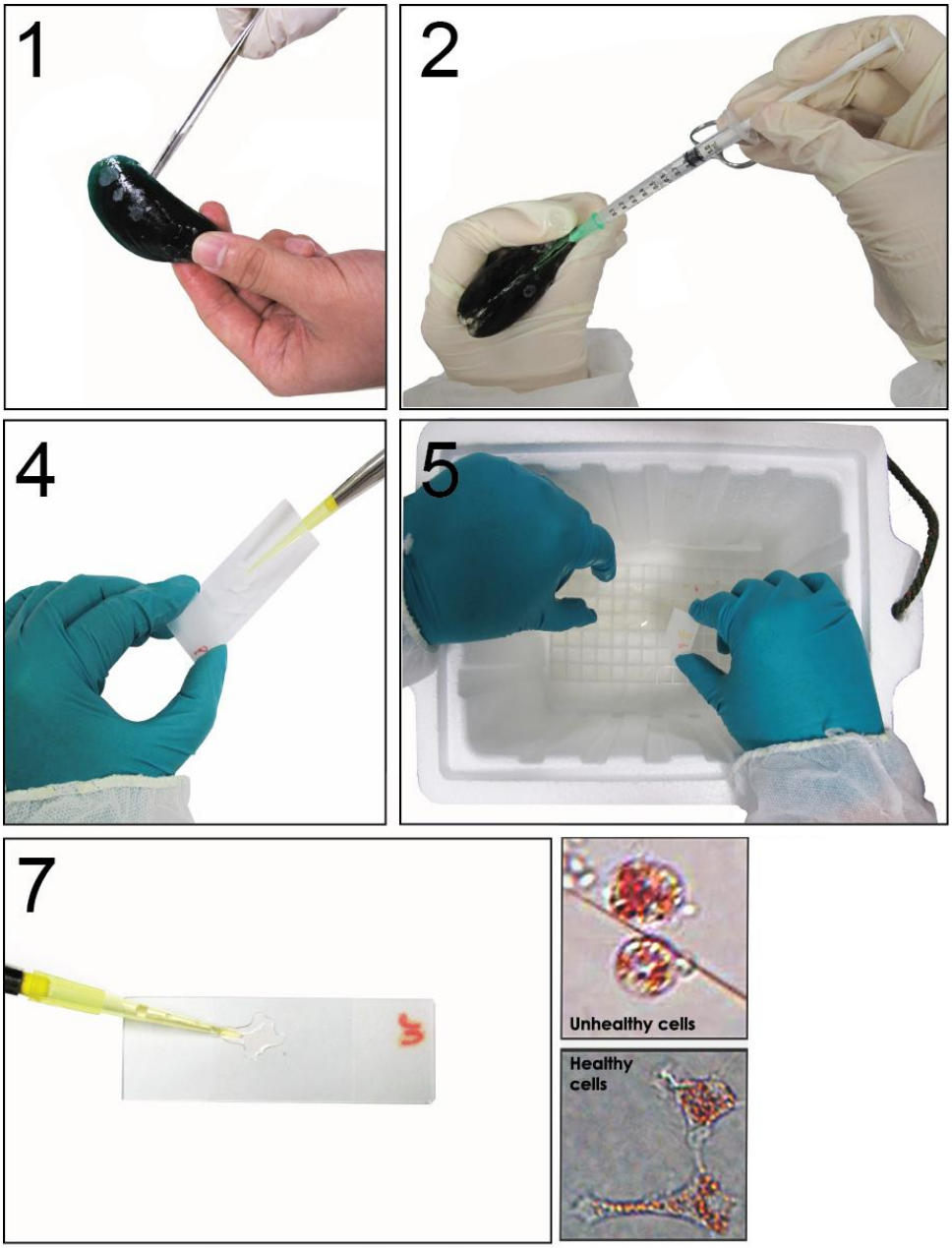
12. Shape of the cells – healthy = irregular; stressed = rounder

13. Size of the cells – healthy = larger; stressed = smaller

14. Colour of the lysosomes – darker = more stressed

15. Size of the lysosomes – larger = more stressed

16. The end point is reached when 50% of the cells in view have released dye from their lysosomes into the cytosol.



The above pictures illustrate procedures involved in steps 1-2, 4-5, 7 on pages 36-37.

E. Phagocytosis assay

Some groups of molluscs (e.g bivalves and gastropods) have an open circulatory system, constantly exposed to varying environmental influences, such as pollutants. Studies (e.g Cole et al. 1995) have demonstrated that some properties of hemocytes (blood cells) obtained from these molluscs are good and sensitive indicators of pollutant stress in the environment. Immune defenses in most bivalves and gastropods are mediated by the phagocytotic action of hemocytes on invading pathogens (Dikkebom et al., 1987). Zymosan A is a pathogen particle conventionally used to assess the phagocytotic integrity of hemocytes. These particles consist of protein-carbohydrate complexes, prepared from the cell wall of the common yeast, *Saccharomyces cerevisiae*. Hemocytes extracted from molluscs are incubated with stained Zymosan A. Thereafter, phagocytosed particles are quantified as a measure of the functionality of the hemocytes. This provides an indication of environmental stressors that may affect the phagocytotic action of hemocytes.

Chemicals and Equipment

- ☑ Marine bivalve physiological saline OR Filtered Sea Water (25ppt FSW)
- ☑ Zymosan A (Sigma Aldrich #Z4250)
- ☑ Ice-cold PBS
- ☑ 2.5% glutaraldehyde OR 4% paraformaldehyde
- ☑ Glycerine OR nail polish
- ☑ 0.05 M Tris-HCl
- ☑ 2% Glucose
- ☑ 2% NaCl
- ☑ 0.5% EDTA
- ☑ 1% Acetic acid
- ☑ 50% Ethanol Zymosan A
- ☑ Neutral Red
- ☑ Baker's formol calcium

Method 1: Counting

1. Withdraw haemolymph from the posterior adductor muscle of the mussel.

2. Place aliquots of 1 – 2 x 10^6 living cells per milliliter on round glass coverslips (10 mm in diameter), located in 24 well microtiter plates. Leave cells to adhere and spread for 45 minutes at room temperature.

3. Alternatively, prepare haemocyte monolayers on plastic Petri dishes (4 cm diameter). Dilute 60 µl-drops of haemolymph with FSW in the ratio of 1:2 (haemolymph:FSW). Diluted haemolymph is used in phagocytosis assays to prevent cell aggregation, to improve the accuracy of enumeration.

4. Leave diluted haemolymph in a moist chamber for about 1h at room temperature to allow haemocytes to adhere and spread on the plastic surface. Rinse haemocyte monolayers at least three times with FSW to remove residual plasma.

5. Add 40 mg of Zymosan A to 10ml of sterile marine bivalve physiological saline or FSW.

6. Heat mixture at 100°C for 30 mins.

7. Spin particles down, and wash twice with saline.

8. Resuspend to a concentration of 1 x 10^8 particles ml^{-1} (or 3.5 x10^7 particles ml^{-1} if following Step 3).

9. Incubate hemocyte monolayers with Zymosan (1: 10; Cell: Zymosan for step 2; OR 60 µl if following step 3) for 90 minutes at 14°C in a humid chamber.

10. Thereafter rinse cells four times with ice-cold PBS or FSW and fix with 2.5% glutaraldehyde or 4% paraformaldehyde.

11. Rinse with saline again, twice.

12. Allow the cover slips to air-dry and mount with glycerine or nail polish.

13. Examine 200 or 400 cells randomly and note the number of hemocytes containing phagocytosed zymosan particles.

Method 2: Using a microplate reader (adapted from Cole et al., 1995)

1. Prepare anticoagulant buffer (0.05 M Tris-HC1, pH 7.6, containing 2% glucose, 2% NaCl and 0.5% EDTA).

2. Extract haemolymph from posterior adductor muscles from the mussels and mix with an equal volume of anticoagulant buffer.

3. To each microplate well, pipette 50 µl of sample.

4. Prepare 1 x 10^8 particles ml^{-1} of neutral red stained, heat stabilized Zymosan suspension (Sigma Chem Co.) in anticoagulant buffer.

5. To each well, also add 50 µl of the Zymosan suspension.

6. Aliquots of Zymosan suspension in anticoagulant buffer alone, and heat-killed cells in Zymosan suspension are used as blanks.

7. Incubate the microplate for 25 min at 10°C.

8. Stop the process by adding 100 µl aliquots of Baker's formol calcium, containing 2% NaCl, to the wells.

9. Allow the cells fix for 10 min, thereafter centrifuge at 70 × g for 5 min and wash several times in buffer.

10. Aliquote suspensions of known Zymosan concentrations (50 µl) into duplicate wells just prior to the last centrifugation to obtain a standard curve.

11. Add Acetic acid (1 %) in 50% ethanol (100 µl) to each well to solubilise the neutral red and allow the plate to stand for 5 min.

12. Read the absorbance at 550 nm and express the results as Zymosan concentration phagocytosed mg^{-1} of haemocyte protein.

F. Cell viability (Eosin Y assay)

Eosin Y is a form of eosin, a red dye commonly used to stain cellular structures such as the cytoplasm and muscle fibers. Eosin Y is used to visualize cell viability after exposure to any contaminant (adapted from Birmelin et al., 1999)

Chemicals and Equipment

- ☑ Cell suspension of interest (prepared previously for e.g netural red assay)
- ☑ 0.05% eosin-Y dissolved in a calcium and magnesium free saline
- ☑ Microscope slides
- ☑ Glass coverslips

Method

1. To a 0.02 ml aliquot of cell suspension, add an equal amount of 0.05% eosin-Y.

2. Mix well and add a drop to a microscope slide. Cover with a coverslip and count the number of stained cells (viable) and non-stained cells (non-viable) at 40x magnification.

G. Fertilization assay

Compared to adult animals, gametes and embryos of marine invertebrates are often more sensitive to environmental pollutants, in particular to metals. Fertilization success and scoring of arrested development in the embryo are assessed here as a bioassay for exposure to heavy metals or other contaminants. As most marine invertebrates are broadcast spawners, exposure of gametes and embryos and tolerance to environmental pollutants becomes a relevant ecological issue.

Chemicals and Equipment

- ☑ Crystallisation dish (150mm x 75mm)
- ☑ 100 ml acid-washed glass beakers
- ☑ Glass Petri dish
- ☑ micropipette (0.5ml, 100µl)
- ☑ Dissecting microscope
- ☑ Potassium dichromate (AppliChem, CAS # 7778-50-9)

Method

1. Induce spawning by placing spawning mussels into the mussel holding tank.

2. Replace spawning mussels from the holding tanks into individual crystallization dishes for collection of gametes as soon as they begin releasing gametes. Females should be kept individually to ensure that all

the eggs collected are unfertilised. Male spawners can be collectively placed in a single crystallization dish.

3. Spawning males are usually observed to release a steady stream of sperm whereas the female spawners generate orange masses of eggs from the exhalent siphons.

4. When the mussels are observed to cease the release of gametes, collect the male and female gametes separately.

5. An egg suspension is made from at least 2 different females. Keep the egg suspension and sperm suspension collected in separate 100 ml beakers.

6. Each test concentration (including controls) should have 5 replicates.

7. A further 5 replicates of unfertilised eggs (unfertilised controls) is included to allow the estimation of the total number of eggs in each test beaker at the start of the test.

8. Add 60 ml of test solution (clean seawater for control and unfertilized control) into each of the test beakers.

9. Add a total of 500 µl volume of egg suspension into each beaker in a randomized order. For each replicate, add 5 aliquots of 100 µl each. Between each 100 µl aliquot, stir the egg suspension to ensure an even distribution of gametes to each test beaker.

10. The test is started by adding 500 µl of sperm suspension to each test beaker.

11. For the 5 unfertilised replicates (unfertilised controls), instead of adding sperm suspension, aliquot 500 µl of 400 mg/l potassium dichromate solution to each test beaker. This is to kill the eggs for an estimation of the number of unfertilised eggs at the start of the test.

12. Incubate the resulting mixtures in ambient conditions after stirring.

13. Score the number of unfertilized eggs after 24 hours.

14. Stir each test beaker before pouring the contents into a glass petri dish.

15. Place the glass petri dish on the stage of a dissecting microscope and count the number of unfertilised eggs in a single field of view (5 mm × 5 mm).

16. A total of 5 counts should be made for each test solution under 5 different fields of view.

17. Repeat this for all the test beakers.

18. Express the total number of unfertilised eggs per beaker as a number per total area of the petri dish (projected estimation).

BIOCHEMICAL ASSAYS

H. Ferric Reducing Antioxidant Power (FRAP)

This assay measures the ability of any biological fluid to delay or prevent oxidation of a oxidizable substrate (Halliwell and Gutteridge, 1999). Within a biological system, a variety of antioxidants such as glutathione, a-tocopherol, ascorbic acid, superoxide dismutase, catalase, peroxidase, etc. are produced within cells to prevent the deleterious effects of potentially harmful reactive oxygen species (ROS).

Under normal conditions, mitochondria are major cellular sources of ROS at between 1% and 3% of their rate of oxygen consumption (Sohal and Weindruch, 1996). As cells undergo stress, ROS will increase with either i) an increase in metabolic rate in stress mediation (Pörtner, 2002) or, ii) in anaerobic respiration causing excess production of oxygen radicals owing to facilitated auto-oxidation of heme groups in mitochondria (Boveris and Chance, 1973). As such, free radical scavenging activities becomes increasingly crucial upon departure from the optimal physiological threshold, and this is indicative of the physiological stress level in animals.

The ferric reducing/antioxidant potential (FRAP) assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. It utilizes the reducing potential of the antioxidants to react with a ferric tripyridyltriazine (Fe^{III}- TPTZ) complex and produce a colored ferrous tripyridyltriazine (Fe^{II}-TPTZ) form (Benzie and Strain, 1996, 1999; Prior and Cao, 1999). The change in absorbance at 593 nm (Halvorsen et al., 2002) can then be compared with a standard curve to determine the antioxidant potential in a given sample. FRAP has been used to analyze antioxidant status in humans after hyberbaric oxygen treatment (Dennog et al., 1999), evaluate patients with chronic

renal failure (Erdogan et al., 2002), compare the effects of different diets on plasma (Lee et al., 2000), examine the influence of dental amalgams on saliva (Pizzichini et al., 2002) and study the efficiency of ascorbate (vitamin C) in plasma (Benzie et al., 1999). It has also been used to compare antioxidant activity in plant and mammalian lignans (Niemeyer and Metzler, 2003) and plant extracts (Tsai et al., 2002), compare the effects of different diets in rats (Aprikian et al., 2001) and evaluate meat quality (Sommers et al., 2003).

Absorbance changes are linear over a wide concentration range with antioxidant mixtures, including plasma, and with solutions containing one antioxidant in purified form. There is no apparent interaction between antioxidants. The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy. The FRAP assay offers a putative index of antioxidant, or reducing potential of biological fluids, and is within the technological reach of every laboratory and researcher interested in oxidative stress and its effects. It should, however, be noted that the FRAP assay does not account for thiol groups in plasma (e.g. glutathione) as they are unable to reduce FE^{III} and should be reported as a total antioxidant status that excludes glutathione (Dissanayake et al., 2011).

Chemicals and Equipment

- ☑ TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) (Sigma Aldrich #T1253)
- ☑ Iron(III) chloride hexahydrate($FeCl_3.6H_2O$) (Sigma Aldrich #F2877)
- ☑ Iron(II) sulfate heptahydrate ($FeSO_4.7H_2O$) (Sigma Aldrich #F7002)
- ☑ Sodium Acetate (Sigma Aldrich #S2889)
- ☑ Glacial Acetic acid (Sigma Aldrich #320099)
- ☑ Micropipettes
- ☑ Microtitre plates
- ☑ Microplate reader (specific wavelength needed: 593 nm)
- ☑ 21 G needles
- ☑ 1ml syringes

Preparation

i) FRAP Reagent

Prepare FRAP reagent fresh as required by mixing the following (A:B:C) in the ratio 10:1:1

(A) 300mM Acetate Buffer

Sodium Acetate	0.775g
Glacial acetic acid	4 ml

Make up to 250ml with distilled water and adjust to pH 3.6

(B) 10mM TPTZ in 40mM HCl

TPTZ	0.078g
1M HCl	1ml

Make up to 25ml with distilled water

(C) 20mM $FeCl_3.6H_2O$

$FeCl_3.6H_2O$ 0.135g

Make up to 25ml in distilled water

ii) Standards: 1mM $FeSO_4.7H_2O$

$FeSO_4.7H_2O$ 0.028g

Make up to 100ml in deionised water

Method

1. Prepare standards (i.e 1mM, 0.5mM, 0.25mM, 0.125mM, 0.063mM, 0.031mM and 0.016mM) via serial dilution using ferrous oxidised iron as follows:
   i. Prepare 8 × 1.5ml Eppendorf tubes and label them **0 to 7**
   ii. Add 500µl of deionized water to **Tubes 0**, **Tubes 2 to 7 ONLY**
   iii. Pipette 1000µl of 1mM $FeSO_4.7H_2O$ into **Tube 1**
   iv. Pipette 500µl of Tube 1's content into **Tube 2**, invert tube to mix
   v. Pipette 500µl of Tube 2's content into **Tube 3**, invert tube to mix
   vi. Continue doing this until **Tube 7** achieves a final volume of 1ml, whereas Tubes 0 to 6 will have a final volume of 500 µl
   vii. Therefore: Tube 1 (**T1**) = 1mM, Tube 2 (**T2**) = 0.5mM, Tube 3 (**T3**) = 0.25mM, Tube 4 (**T4**) = 0.125mM, Tube 5 (**T5**) = 0.063mM, Tube 6 (**T6**) = 0.031mM, Tube 7 (**T7**) = 0.016mM, Tube 0 (**T0**) = Blank

2. At this point, configure the microtiter plate reader using the above concentration values, and the photograph on page 54 to help you.

3. Remove approx 100µl of haemolymph from each mussel and place on ice. (Tip: As there are 96 wells in a microtiter plate, a maximum of 48 mussels can be examined for this assay)

4. Centrifuge haemolymph for 5 mins at 1000rpm.

5. Prepare the FRAP reagent by mixing the following together :
   10mls of 300mM Acetate buffer
   1ml of TPTZ
   1ml of 20mM Iron Chloride

6. Add 50µls of each standard to two wells (two replicates), by the photograph on page 54.

7. Add 50µls of plasma (not haemocytes therefore use top layer of centrifuged solution) to two wells for each mussel sample, as indicated by the photograph on page 54 (S1 = Mussel Sample 1; S2 = Mussel Sample 2 and so on).

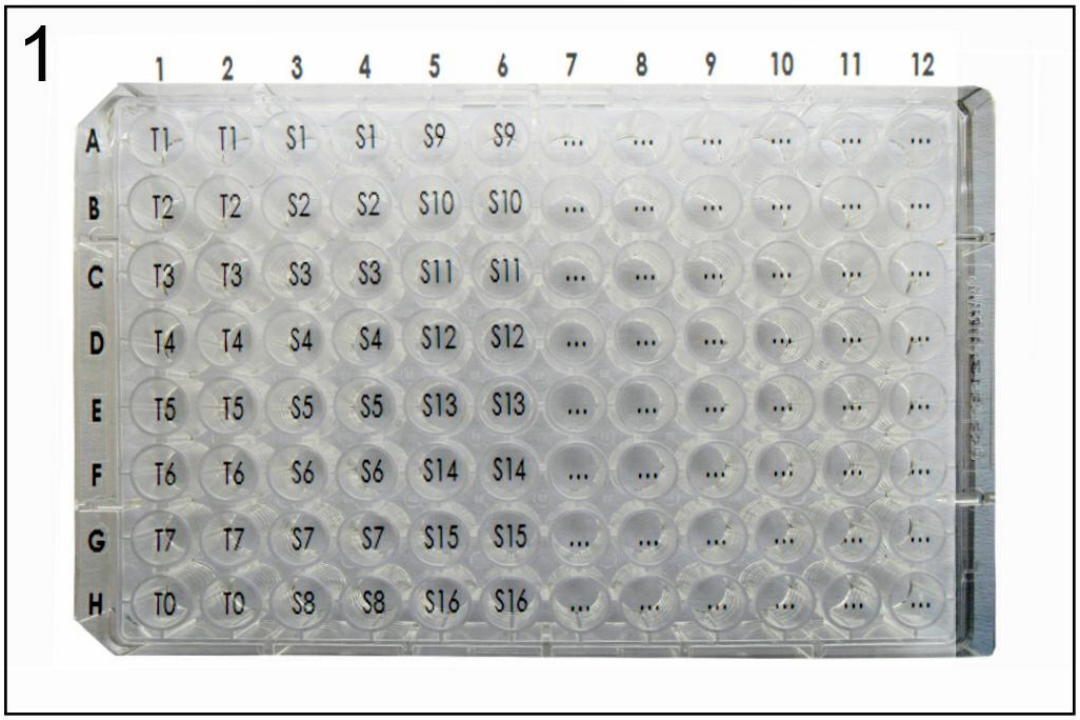
8. Place 200µl of FRAP reagent into each well.

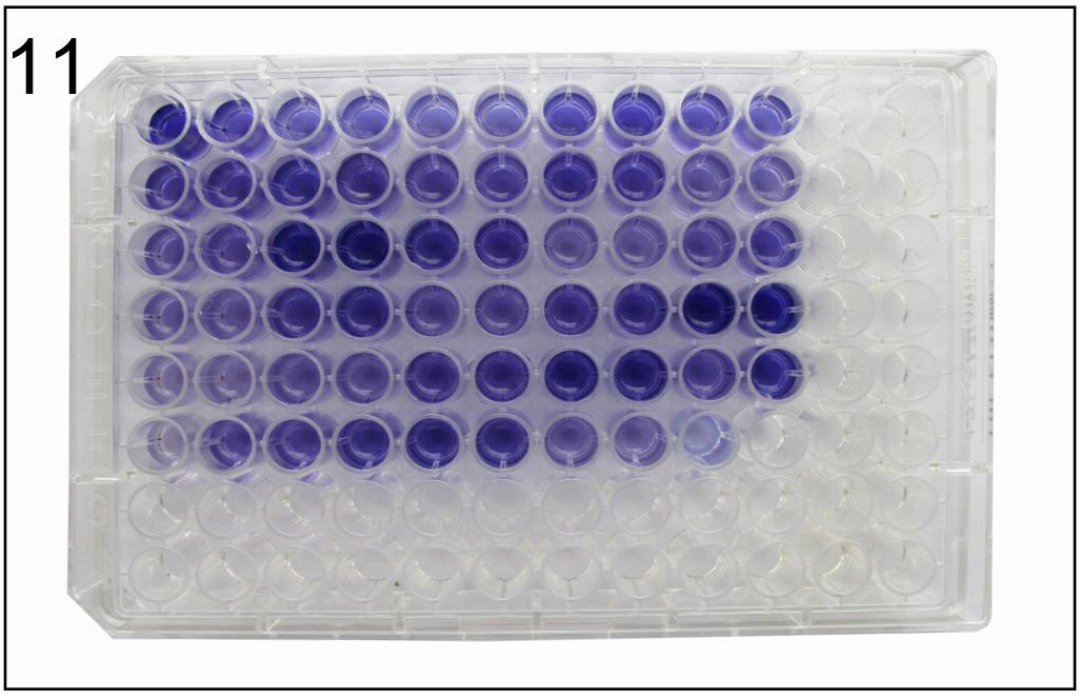
9. Place plate into the plate reader.

10. Incubate in the machine for 10 minutes at 25˚C.

11. Read absorbance at 593nm.

Note 1: A darker colour indicates more anti-oxidants present.
Note 2: The results should increase over time.





The above pictures illustrate procedures involved in steps 1 and 11 on pages 52-53.

I. Glycogen analysis (Anthrone assay)

Glycogen serves as a secondary long term energy store in animal cells and can be used as a bioindicator of stress levels and energy reserves (Leung and Furness, 2001; Palais et al, 2011) representing the readily mobilisable storage form of glucose for most organisms. Although analysis of adenylate energy charge (assessment of Adenosine-5'-triphosphate levels) would be the most direct and accurate gauge of the readily available energy reserve of an individual, the assay presented here using Anthrone (a tricyclic aromatic ketone popularly used in the colorometric determination of carbohydrates) is a lot easier and faster to manipulate (Sattler and Zerban, 1948).

Here the assay is modified from that described by Seifter et al (1950) but remains the same in terms of chemical processing of the sample: the tissue is digested in hot concentrated KOH solution and glycogen precipitated with ethanol, followed by hydrolysis of the precipitate with mineral acid, and determination of the glucose in the hydrolyzate as reducing sugar. This method was made quantitative and was suggested for the analysis of glycogen by Morris (1948) with the anthrone reagent. It was postulated that the sulfuric acid medium of the reagent causes dehydration of the sugar to a furfural derivative, which then condenses with anthrone to form a blue-coloured compound which can be measured using a spectrophotometer.

Chemicals and Equipment

- ☑ Potassium hydroxide (pastilles) (Sigma Aldrich #P1767)
- ☑ D-(+)-Glucose (Sigma Aldrich #G5767)
- ☑ Anthrone (Fluke #10740)
- ☑ Ethanol (Sigma Aldrich #493538)
- ☑ Micropipettes
- ☑ Waterbath (100˚C)
- ☑ Microcentrifuge
- ☑ Fume hood
- ☑ Deionized water (in ice)

Method

1. Prepare 30% w/v Potassium hydroxide solution by dissolving the hydroxide pellets in deionized water at a ratio of 30g pellets to 100ml water. Prepare enough for tissue digestion (400µl per sample to digest). Always store the hydroxide in plastic bottles as the strong alkali will etch glass containers.

2. Add 72ml of conc. H_2SO_4 **SLOWLY** to 28ml of water (kept in ice). Always add acid to water and not vice versa.

3. Allow the solution to cool to room temperature and dissolve 50mg of Anthrone into the acid.

4. Store the Anthrone solution at 0~5˚C until ready to use in step 16.

5. Excise the tissue to be analysed into pre-weighed eppendorf tubes and note the wet weight of the tissue.

6. To each 50mg wet weight sample (5mg dry weight) add 400µl of 30% w/v Potassium hydroxide.

7. Boil the sample in a waterbath (100˚C) for at least 20 minutes until the tissue dissolves.

8. Add 1ml of absolute alcohol to each eppendorf tube.

9. Cool the samples on ice for 2 hours.

10. Centrifuge the eppendorf tubes at 40,000rpm for 10 mins.

11. Discard the supernatant (in the fume hood).

12. Add 1ml of deionized water to each eppendorf tube.

13. Take 50µl for analysis.

14. Prepare the glucose standard using either glucose or glycogen:

	5mM	**2.5mM**	**1mM**	**0.5mM**
D-glucose	90mg.100ml^{-1}	45mg.100ml^{-1}	18mg.100ml^{-1}	9mg.100ml^{-1}
Glycogen	81 mg.100ml^{-1}	40.5mg.100ml^{-1}	16.2mg.100ml^{-1}	8.1mg.100ml^{-1}

15. Take 50µl from each standard for analysis too.

16. To each glucose standard and sample, add 1ml of Anthrone reagent.

17. Boil the eppendorf tube at 100˚C for 10 mins.

18. Read the absorbance of the resulting fluid with a spectrophotometer at 620nm.

19. Plot the standard curve from the readings obtained with the glucose standards and work out the glycogen level of each sample analysed.

20. Divide the glycogen level of each sample by its wet/dry weight.

J. Heat shock protein (Hsp70)

In 1962, it was discovered that sublethal heat shock would induce a RNA transcription in the fruit fly *Drosophila melanogaster* (Ritossa, 1962). These inductions were very rapid, occurring within a minute of the heat shock, but regressed only after 30 to 40 minutes. The RNA transcribed codes for a protein translation, which was termed heat shock protein (hsp). Originally associated with thermal stress, it was later discovered that a wide spectrum of stressors such as trace metals (Sanders, et al., 1991; Bauman et al., 1993; Williams et al., 1996), organic pollutants (Sanders, 1990), osmolarity (Klutz, 1996), hypoxia and anoxia (Mestril et al, 1994; Myrmel e al., 1994) and exposure to ultraviolet radiation (Nepple and Bachofen, 1997) could all induce production of hsp, and the broader terms of "cellular stress response" and "stress protein" were thus coined (Goering, 1993). HSPs are ubiquitous, highly conserved proteins, believed to assist in a variety of cellular processes such as protein translocation, folding of newly translated proteins, regulation of apoptosis, refolding of damaged proteins, targeting severely damaged proteins to be degraded and in the case of excessive damage, sequestering damaged proteins to larger aggregates for ease of cellular degradation (Hartl, 1996). Of these protein families, Hsp70 has the largest specific activity, or amount produced in a given time, of all the stress proteins, and thus is an easier protein to detect. It is also highly conserved, and has been identified in archaeabacteria, several species of algae, many marine invertebrates and all the classes of chordates (see Feder and Hofmann, 1999 for review). Under adverse environmental conditions the synthesis of hsp70 increases and they act to repair and protect cellular proteins from stressor-induced damage and also minimize protein aggregation. Thus, an increase in the total specific activity of hsp70 within a biological system can be used as a non-specific indicator of stress.

Traditionally, hsp70 is detected and quantified by Western Blot (protein immunoblot) analysis which in gist involves using the SDS-PAGE (sodium dodecyl

sulfate polyacrylamide gel electrophoresis). The technique is a one dimensional separation of proteins by molecular weight and subsequent binding (staining) with antigen-specific antibodies tagged with an appropriate substrate which drives a colourimetric/fluorescence reaction. The intensity of this reaction is correlated with the concentration of the protein detected. For simplicity, hsp70 is detected here using a ELISA (Enzyme-linked immunosorbent assay) commercial kit. In summary, antigens from the sample are attached to a solid surface. Then, antibody specific to hsp70 is applied over the surface so it can binds to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, a color change, in the substrate and quantification of the protein read over an absorbance wavelength.

Chemicals and Equipment

- ☑ Quick Start Bradford Protein Assay kit 1 (Bio-Rad #500-0201)
- ☑ ENZO HSP70 ELISA Kit (ENZO (vCell) #ADI-EKS-700B)
- ☑ Protease inhibitor cocktail (Fluke # 20-201 OR Sigma Aldrich #S8820)
- ☑ Micropipettes
- ☑ Microplate reader (capable of 590 and 420nm wavelength)
- ☑ Microcentrifuge (refrigerated)
- ☑ Eppendorf tubes
- ☑ Tissue disruptor/homogenizer (optional if no liquid nitrogen is available)
- ☑ Mortar and pestle

Method

As detailed methods are included in the ELISA kit, the method here just covers protein extraction and total protein assessment.

1. As this particular ELISA kit includes an extraction stock, dilute the required amount of extraction buffer with pre-chilled deionized water, following instructions in the ELISA kit.

2. For better integrity of data acquisition, commercial protease inhibitor cocktails should be added to the extraction buffer according to the instructions provided by the cocktail manufacturer.

3. If a commercial cocktail tablet is not available, examples of appropriate protease inhibitors include 0.1mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin, 1µg/ml pepstatin.

4. Keep this extraction buffer chilled (4˚C).

5. For each ~ 0.5cm^3 piece of tissue, use 1ml of 1× Hsp70 extraction buffer.

6. After incubation of treated mussels, remove the mussel from the treatment tank and using a long pair of forceps quickly dip the animal into liquid nitrogen to kill the animal.

7. Gently pry open the shells and quickly excise the target tissue to be analysed. Potential candidates include the gills or foot muscle. Avoid using whole animal tissue as there are different degress of hsp induction in different body parts, and this will compromise the protein reading. This should be done in a cold room, or on ice, if possible, to keep the temperature of the tissue as low as possible.

8. If necessary, tissues can be flash frozen, stored at -70°C and the extract prepared at a later time.

9. Place the tissue in a mortar and add a sufficient volume of liquid nitrogen to cover the tissue.

10. Allow the liquid nitrogen to evaporate. The tissue should be thoroughly frozen.

11. Grind the frozen tissue to a powder with a pestle. Add an appropriate volume of 1× extraction buffer supplemented with protease inhibitors to the processed tissue.

12. Continue to homogenize the tissue with the pestle until the tissue suspension is homogeneous.

13. Transfer the extract to a polypropylene tube and centrifuge at 21,000 × g for 10 minutes in a 4°C refrigerated microfuge.

14. Transfer the supernatant to a labelled polypropylene tube. The supernatant collected is the tissue extract, which is now ready for analysis.

15. Repeat this for all the samples to be analysed.

16. A Bradford test should be performed to determine the total protein content of each tissue extract.

17. A Bio-Rad Bradford Protein kit is used here using a microassay protocol:

    i. The linear range of this assays with Bovine albumin is 1.25–10 µg/ml.

    ii. Remove the 1× dye reagent from the 4°C storage and let it warm to ambient temperature. Invert the 1× dye reagent a few times before use.

    iii. Dilute the standards using the table below.

    iv. Pipette 150µl of each standard and unknown sample solution into separate microplate wells. Add 150µl of 1× dye reagent to each well and mix the samples using a microplate mixer. Alternatively, use a multichannel pipette to dispense the 1× dye reagent. Depress and release the plunger repeatedly to mix the sample and reagent in the wells. Replace with clean tips and add reagent to the next set of wells.

    v. Incubate at room temperature for at least 5 min. Samples should not be incubated longer than 1 hr at room temperature.

vi. Set the absorbance wavelength to 595 nm. Measure the absorbance of the standards, blanks, and unknown samples.

Tube #	Standard volume (µl)	Source of standard	Diluent volume (µl)	Final protein (µg/ml)
1	10	2mg/ml stock	790	25
2	10	2mg/ml stock	990	20
3	6	2mg/ml stock	794	15
4	500	Tube 2	500	10
5	500	Tube 4	500	5
6	500	Tube 5	500	2.5
7	500	Tube 6	500	1.25
8 (blank)	-	-	500	0

K. Total Glutathione assay

Glutathione (L-g-glutamyl-L-cysteinylglycine) is the principal non-protein thiol involved in the antioxidant cellular defence. It is a tripeptide composed of cysteine, glutamic acid and glycine, and its active group is represented by the thiol (–SH) cysteine residue. In cells, total glutathione can be free or bound to proteins. Free glutathione is present mainly in its reduced form, which can be converted to the oxidised form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase. The redox status depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG) and appears to be a critical determinant in the cell. In normal conditions, the glutathione redox couple is present in mammalian cells in concentrations between 1 and 10 mM, with the reduced GSH predominating over the oxidised form. In the resting cell, the ratio exceeds 100, whereas in various models of oxidative stress, this ratio was reported to decrease to values between 10 and 1 (Chai et al., 1994).

The Glutathione (total), detection kit is a complete kit for the measurement of total, reduced, and oxidized glutathione concentrations in cell and tissue extracts, whole blood, plasma, saliva, and urine. Increased oxidative damage of proteins, lipids, and DNA by free radicals is one of the pathogenic mechanisms of diseases.

Glutathione, a major intracellular non-protein thiol, protects against free radical damage by providing reducing equivalents for several key antioxidant enzymes. In addition Glutathione acts as a scavenger of hydroxyl radicals and nascent oxygen. Elevated Glutathione levels have been associated with a reduced rate of illness by providing a primary defense system for the removal of oxidants.

The concentration of Glutathione ranges from 10µm to 1mM in cells and is in the micromolar range in plasma. The Total Glutathione Assay kit described here

(Enzo-Life Science Total Glutathione Assay kit ADI-900-160) utilizes a carefully optimized enzymatic recycling method for the quantification of glutathione. Glutathione Reductase reduces oxidized glutathione (GSSG) to reduced glutathione (GSH). As shown in Figure 1, the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoicacid, Ellman's reagent) to produce a yellow colored 5-thio-2-nitrobenzoic acid (TNB) that absorbs at 405 or 414 nm. The rate of TNB production is directly proportional to the concentration of glutathione in the sample. The measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of glutathione in the sample.

This particular assay complements the FRAP assay (Section H) covered in the earlier sections as the thiol group does not react with the FE^{III} substrate in the FRAP assay. Hence the same mussel haemolymph sample may be used for both assays.

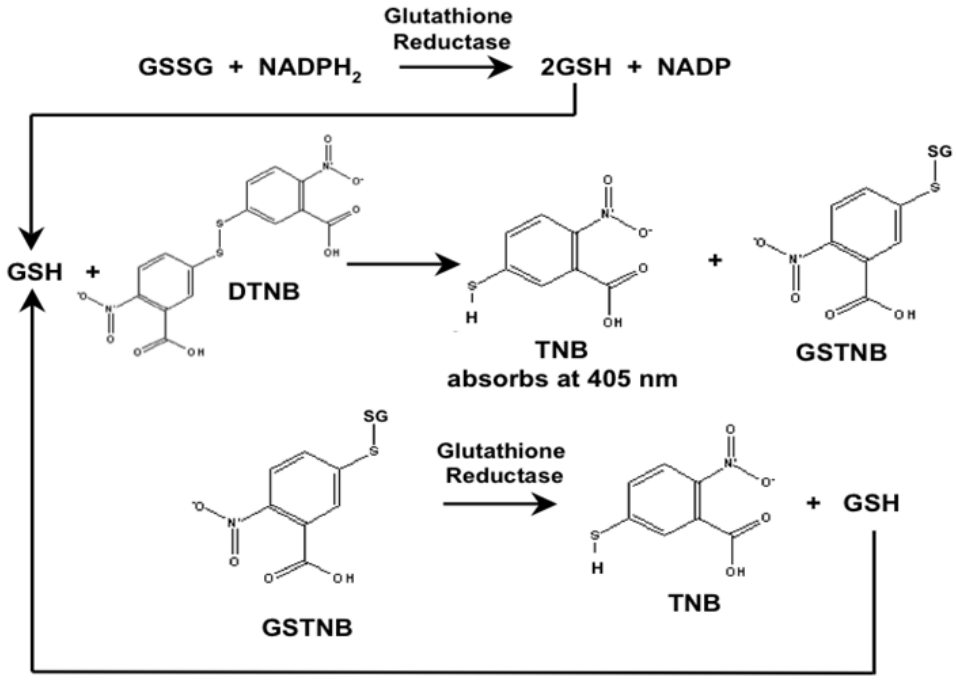


Figure 1. Schematic chemical reaction of the Total Glutathione Assay (adapted from manual of Enzo Life Sciences product catalog #ADI-900-160).

Chemicals and Equipment

- ☑ Enzo Life Science Total Glutathione Assay kit #ADI-900-160
- ☑ Phosphate Buffer Solution
- ☑ Distilled water
- ☑ Metaphosphoric Acid (Aldrich Catalog No. 23927-5)
- ☑ 4-Vinylpyridine (Aldrich Catalog No. V3204-5ML)
- ☑ Reagent alcohol
- ☑ Microtubes, 0.5 and 1.5 mL
- ☑ 15mL conical tubes (adherent and suspension cell preparation)
- ☑ 50 mL conical tubes (tissue preparation)
- ☑ Precision pipettes for volumes between 5-1000 µL
- ☑ Multichannel pipettor for volumes between 1-50 µL and 50 µL-200 µL
- ☑ Microplate reader capable of reading at 405 or 414 nm and taking readings every minute for ten minutes and exporting data to an Excel spreadsheet
- ☑ Centrifuge and microfuge for processing samples
- ☑ Sonicator or Homogenizer

Preparation

i) 1× Assay Buffer

Allow the assay buffer to come to room temperature for a minimum of 30 minutes. Dilute the 25× Assay Buffer to 1× (1:25) with distilled water. The 1× Assay Buffer is used to prepare dilutions for the Total Glutathione Standard Curve, and to dilute each experimental sample.

ii) Reaction Mix

Reconstitute one or more bottles of Reaction Mix with 8 mL of distilled water. Periodically swirl the bottle gently over a 15 minute period to dissolve contents. Immediately before use in the assay, vortex the vial of Glutathione Reductase and add 10 µL of the Glutathione Reductase to the bottle of Reaction Mix. Each bottle of Reaction Mix is sufficient for 53 wells in a 96-well plate, or little more than half a plate. Pool the reconstituted Reaction Mix together into one tube if more than one bottle is used.

iii) 5% (w/v) Metaphosphoric acid

Prepare 5% (w/v) Metaphosphoric acid in distilled water (not included in kit).

iv) 2M 4-Vinylpyridine

Prepare 2M 4-vinylpyridine solution by mixing 108µL 4-vinylpyridine with 392µL ethanol (solution should be prepared and subsequently used only in a chemical fume hood).

Use immediately and discard any unused portion.

Note: It is recommended that you use 4-vinylpyridine within 1 month of purchase and store at -20ºC. This reagent blocks free thiols present in the reaction, thus eliminating any contribution to the cycling reaction caused by GSH.

v) Experimental Samples

After preparing the samples as outlined in the Tissue Handling section that follows, dilute each sample 1:10 with 1× Assay Buffer. Some samples such as whole blood, liver, or red blood cells, may need to be diluted 1:20, 1:40, or more. It may

be necessary to make serial dilutions of your extracts to obtain a satisfactory change in absorbance readings with time.

Tissue Handling

All samples are treated with 5% (w/v) Metaphosphoric acid to remove proteins which interfere with the assay.

i) Cell Lysate Preparation

1. Detach adherent cells by gentle trypsinization. Count the cells and centrifuge at 300 x g for 10 minutes at 4°C. Wash the cells once with cold 1× PBS. Centrifuge suspension cells at 300× g for 10 minutes at 4ºC.

2. Discard the supernatant. Wash the cells once with cold 1× PBS.

3. Suspend the pellet with 500 µL of cold 5% (w/v) Metaphosphoric acid per 2-5 × 106 cells. Mix thoroughly by repeated pipetting. Homogenize or sonicate the cell suspension and store on ice for 5 minutes. Transfer the suspension to a 1.5 mL tube and centrifuge at 12,000-14,000 × g for 5 minutes at 4ºC. Place the supernatant into a clean 1.5 mL tube. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

ii) Tissue Lysate Preparation

1. Remove tissue. Repeatedly wash the tissue with cold isotonic saline (150 mM) or 1× PBS with 0.16 mg/mL heparin to prevent coagulation.

2. Blot tissue on filter paper and weigh.

3. Add ice-cold 5% (w/v) Metaphosphoric acid (20 mL/g tissue) and homogenize using a cold glass or teflon pestle.

4. Centrifuge the homogenate at 12,000-14,000 × g for 10-15 minutes at 4°C.

5. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

iii) Erythrocyte Lysate Preparation

1. Collect blood in Vacutainers containing heparin or sodium citrate as anticoagulant. Centrifuge at 3,000 × g for 10-15 minutes at 4ºC.

2. Discard as much of the plasma supernatant as possible.

3. Remove the white buffy coat (leukocytes) on the surface of the erythrocytes.

4. Resuspend the erythrocyte pellet in four volumes of ice cold 5% (w/v) Metaphosphoric acid. Mix thoroughly and store on ice for 15 minutes.

5. Centrifuge the suspension at 12,000-14,000 × g for 10-15 minutes at 4°C.

6. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

iv) Whole Blood Lysate Preparation

1. Collect blood in tubes containing heparin or sodium citrate as anticoagulant.

2. Add four volumes of ice-cold 5% (w/v) Metaphosphoric acid. Mix thoroughly and store on ice for 15 minutes.

3. Centrifuge at 12,000-14,000 × g for 10-15 minutes at 4ºC.

4. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

v) Urine, Plasma, and Saliva Preparation

1. Collect urine, plasma, or saliva and immediately add four volumes of ice-cold 5% (w/v) Metaphosphoric acid. Mix thoroughly and store on ice for 15 minutes.

2. Centrifuge at 12,000-14,000 × g for 10-15 minutes at 4ºC.

3. Collect the clarified supernatant. Store on ice if you intend to immediately assay for Glutathione, or freeze at -80°C for future use.

Method

I) Total Glutathione Assay

1. Set up the Glutathione standard curve:

   i. Add 50 µL of 1× Assay Buffer to all the wells in rows A through E, columns 1 and 2 of the microtiter plate.

   ii. Add 50 µL of the 4 µM GSSG to wells A1 and A2. Mix well by pipetting the solution up and down at least ten times.

   iii. Transfer 50 µL from wells A1 and A2 to wells B1 and B2 respectively. Mix well at least 10 times and transfer 50 µL from row B to row C. Continue in this fashion to row D. Mix and discard the last 50 µL from row D. Wells E1 and E2 are set aside as Blank wells. The GSSG content in rows A, B, C, and D, is 100 pmoles/well, 50 pmoles/well, 25 pmoles/well, and 12.5 pmoles/well, respectively.

2. Add 50 µL of your diluted experimental samples to the wells in columns 3 to 12. Note: It may be necessary to make serial dilutions of your extracts to obtain a satisfactory change in absorbance readings with time, but for this workshop we will use a 10× dilution factor.

3. Prior to the next step, set up the parameters of your plate reader to measure absorbance at 405 nm or 414 nm and to read the required wells. Include a 10 second orbital shake prior to the initial read.

4. Add 150 µL of freshly prepared Reaction Mix to each well.

5. Immediately record the absorbance in the wells at 405 nm or 414 nm using a plate reader at 1 minute intervals over a 10 minute period. Note: If you intend to use all the wells on one plate in the assay, it may be necessary to record the absorbance at 2 minute intervals.

II) Oxidized Glutathione Assay

1. Add 1µL of 2M 4-vinylpyridine per 50µL of sample and 4µM GSSG. Incubate for one hour at room temperature (cell lysates should be diluted at least 1:10 prior to 4-vinylpyridine treatment).

2. Serially dilute the 4-vinylpyridine-treated GSSG standard as described above in the total glutathione assay protocol (Step 1).

3. Serially dilute your 4-vinylpyridine-treated experimental samples as described above in the total glutathione assay protocol (Step 2).

4. Follow steps 3, 4, and 5 as described above for the total glutathione assay.

Calculation of Results

I) Determination of Total Glutathione Concentration

1. Take the average of the triplicate absorbance readings for each standard, sample, and Blank at each time point.

2. Plot the average of each standard, sample, and background absorbance (A405 nm) versus incubation time and determine the slope from the linear portion of each curve (Figure 2).

3. Subtract the background slope from the slopes of the standards and the experimental samples.

4. Plot the net slopes of the GSSG standards versus pmoles of Glutathione (Figure 3).

5. Compare the net slopes of the experimental samples with those of the standard curve from Figure 3 to determine the pmoles of GSSG (equivalent to total glutathione) for each experimental sample.

II) Determination of Oxidized Glutathione Concentration

1. Follow the procedure described above for generating the Standard GSSG curves (Figures 2 and 3) for the 4-vinylpyridine treated standards.

2. Compare the net slopes of the 4-vinylpyridine-treated experimental samples with those of the 4-vinylpyridine treated standard curve from Figure 3 to determine the pmoles of oxidized Glutathione for each experimental sample.

3. Subtract the pmole of oxidized glutathione in your sample from the pmole of total glutathione to obtain the pmole of reduced glutathione in your sample.

Reduced GSH = Total glutathione − Oxidized GSSG

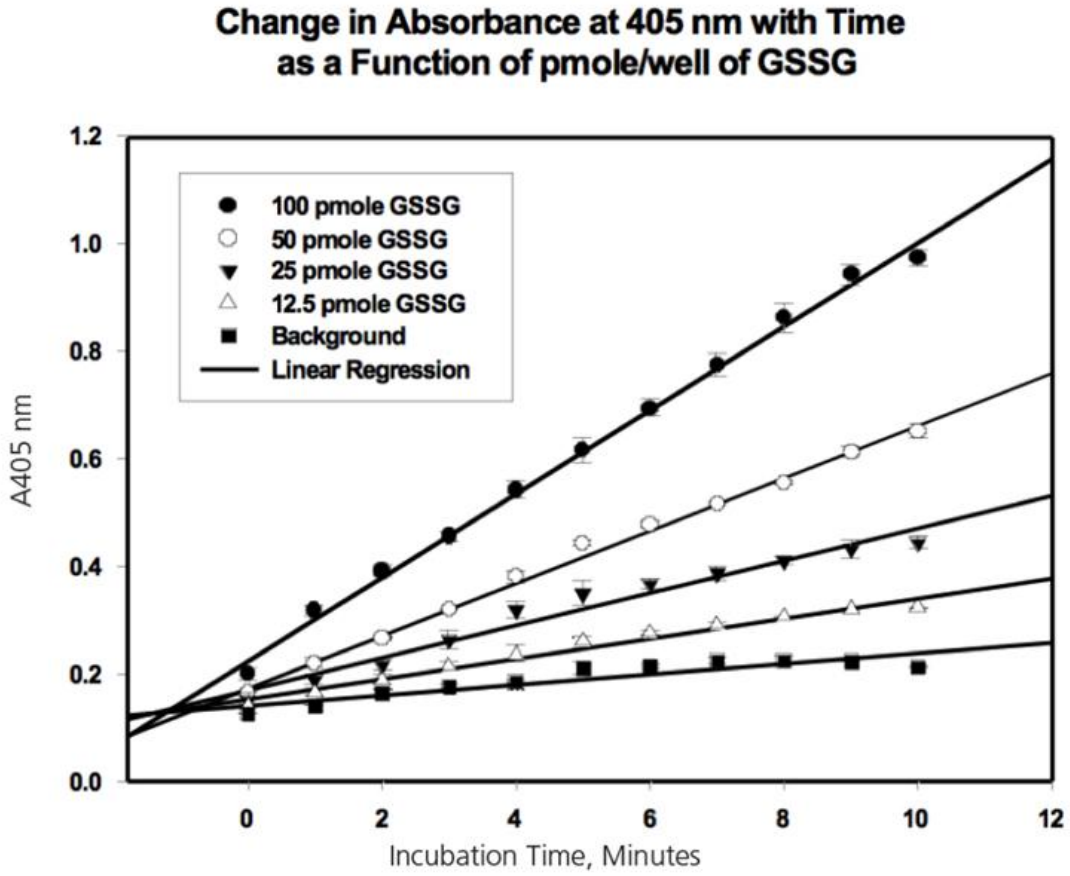


Figure 2. Plot of absorbance at 405 nm versus incubation time as a function of pmoles of GSSG/well (from manual of Enzo Life Sciences product catalog #ADI-900-160).

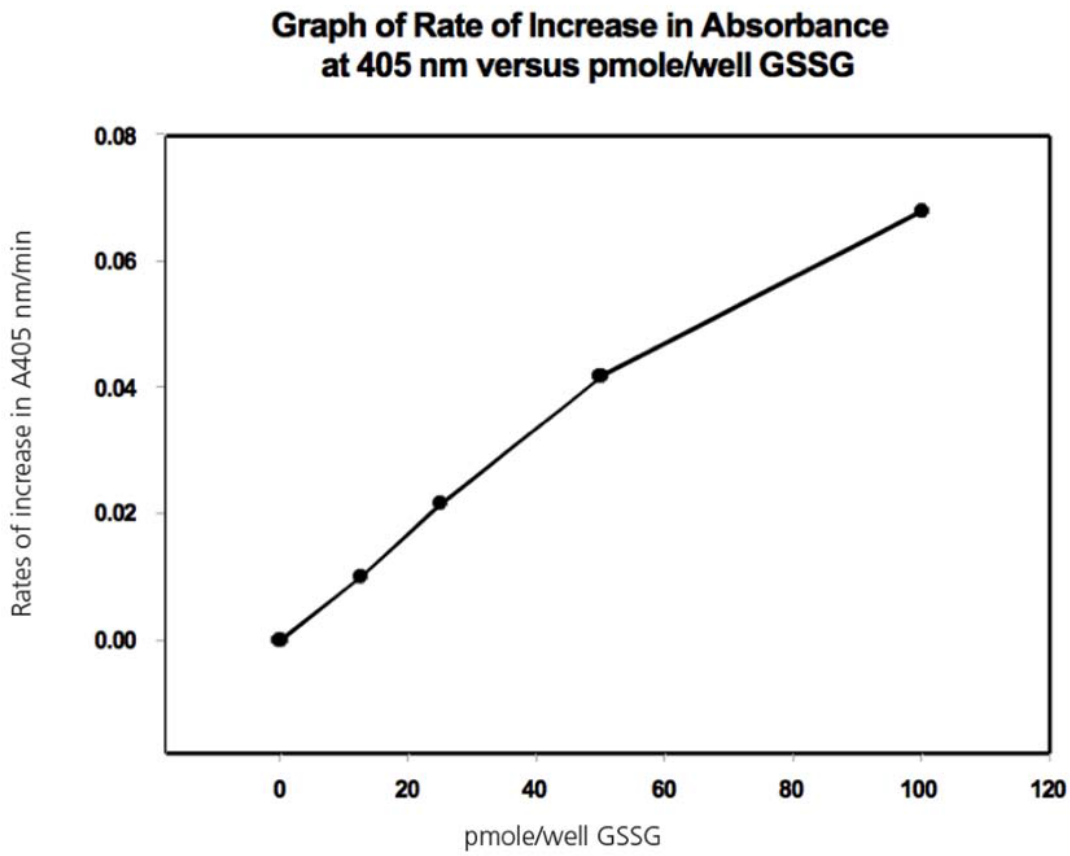


Figure 3. Rate of increase in the absorbance at 405 nm as a function of pmole/well GSSG (from manual of Enzo Life Sciences product catalog #ADI-900-160).

MOLECULAR ASSAYS

L. Genotoxicity: Single Cell Gel Electrophoresis

As opposed to the other assays covered in this manual that reflect integrated stress responses, the Single Cell Gel Electrophoresis assay specifically measures DNA damage and genotoxicity and is thus only viable for the assessment of environmental stressors that affect DNA integrity (Singh et al., 1988). Alternatively known as the comet assay, the name originates from the comet-like pattern of DNA migration through an electrophoresis gel, giving an appearance resembling a comet (Figure 4).

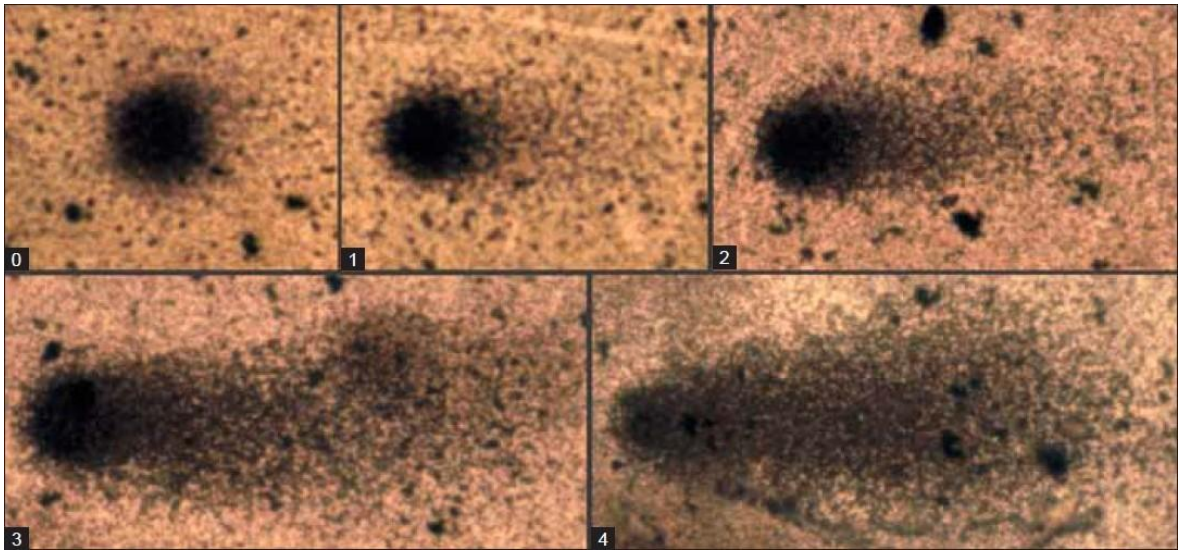


Figure 4. Silver staining of an eukaryotic cell. The length of the comet tail and disintegration of comet head is proportional to the amount of DNA damage.

The principle of the technique involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells (see Collins and

Azqueta, 2012). The likely basis for this is that DNA loops containing a break lose their supercoiling and become free to extend toward the anode. This is followed by visual analysis after staining of DNA, to determine the extent of DNA damage using the length of the comet tail and/or the disintegration of the comet head. This can be performed by manual scoring or automatically using imaging software. For this demonstration, silver staining of DNA is adopted to replace the traditional and highly toxic ethidium bromide stain.

The comet assay is an extremely sensitive DNA damage assay. This sensitivity means that the sample needs to be handled carefully as it is also vulnerable to physical changes which may affect the reproducibility of results. Essentially, anything that can cause DNA damage or denaturation except the factor(s) being researched should be avoided. Although both alkaline and neutral electrophoresis may be performed depending on the type of DNA damage being investigated, the most common form of the assay is the alkaline version (both versions are covered in this handbook). Due to its simple and inexpensive setup, it can be used in conditions where more complex assays are not available. Finally, if agarose gel electrophoresis equipment are not readily available, Ens et al (2012) provides a suggestion for setting up the experiment with household materials for DNA gel electrophoresis.

Chemicals and Equipment

- ☑ Powerpac for electrophoresis
- ☑ Submarine electrophoresis tanks
- ☑ Trevigen Comet Assay® Silver Reagent kit (Cat # 4251-050-K)
- ☑ Haemocytometer
- ☑ Micropipette (capable of 20~200µl aliquots)
- ☑ Compound microscope
- ☑ Phosphate buffer solution
- ☑ 95% Ethanol
- ☑ TE Buffer
- ☑ NaOH pellets
- ☑ EDTA
- ☑ Methanol
- ☑ Glacial Acetic Acid

The electrophoresis conditions used will determine the sensitivity of the assay. The Neutral CometAssay® detects double-stranded DNA breaks, while the Alkaline CometAssay® detects single and double-stranded DNA breaks, and the majority of abasic sites as well as alkali labile DNA adducts (e.g. phosphoglycols, phosphotriesters). The Alkaline CometAssay® requires approximately 2–3 hours to complete whereas the Neutral CometAssay® requires 4 hours, including incubation times and electrophoresis. Once the cells or tissues have been prepared the procedure is not labor intensive. The Lysis Solution may be cooled and the LMAgarose melted while the cell and tissue samples are being prepared. When dealing with a large number of samples, a convenient stopping point is to perform cell lysis overnight (Alkaline step 5). In addition, cryopreservation allows experimental samples to be processed concurrently.

Preparation: Reagents for All Molecular Assays

i) 1× PBS, Ca^{++} and Mg^{++} free

Dilute 10× PBS with deionized water to prepare 1× PBS and store at room temperature (10× PBS is available from Trevigen, cat# 4870-500).

ii) Lysis Solution

For up to 10 slides (2 samples per slide) prepare:

Lysis Solution (cat# 4250-050-01)	40 ml
DMSO (optional)	4 ml

Cool to 4°C for at least 20 minutes before use. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. The buffer formulation is proprietary.

iii) Comet LMAgarose

The Comet LMAgarose is ready to use once molten. Loosen the cap to allow for expansion then heat the bottle in a 90-100°C water bath for 5 minutes, or until the agarose is molten (Caution: Microwaving is not recommended). Place the bottle in a 37°C water bath for at least 20 minutes to cool. The LMAgarose will remain molten at 37°C for sample preparation indefinitely. The LMAgarose formulation is proprietary.

Preparation: Alkaline Comet Assay

i) Alkaline Unwinding Solution, pH>13 (200 mM NaOH, 1 mM EDTA)

NOTE: Wear gloves when preparing and handling the Alkaline Unwinding Solution.

Per 50 ml of Alkaline Solution combined:	
NaOH Pellets	0.6 g
200 mM EDTA (cat # 4250-050-04)	250 µl
dH_2O	49.75 ml

Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

ii) Alkaline Electrophoresis Solution pH >13 (200 mM NaOH, 1 mM EDTA) for the CometAssay® ES:

Prepare a stock solution of 500 mM EDTA, pH 8.

For 1 liter of electrophoresis solution:	
NaOH pellets	12 g
500 mM EDTA, pH 8	2 ml
dH_2O (after NaOH is dissolved) add to:	1 liter

Use of freshly made solution is recommended. Cool to 4°C.

Preparation: Neutral Comet Assay

i) 1× Neutral Electrophoresis Buffer

To prepare 10× Neutral Electrophoresis Buffer:

Tris Base (mol. wt. = 121.14)	60.57 g
Sodium Acetate (mol. wt. = 136.08)	204.12 g

Dissolve in 450 ml of dH_2O. Adjust to pH = 9.0 with glacial acetic acid. Adjust volume to 500 ml, filter sterilize and store at room temperature. Dilute the 10× stock to 1× in dH_2O to prepare 1 liter working strength buffer and cool to 4°C.

ii) DNA Precipitation Solution

Prepare a 10 ml stock solution of 7.5M Ammonium Acetate:

NH_4Ac (mol. wt. = 77.08)	5.78 g
dH_2O (after NH_4Ac is dissolved) add to:	10 ml

For 50 ml of DNA precipitation solution combined:

7.5 M NH_4Ac (mol. wt. = 77.08)	6.7 ml
95% Ethanol (reagent grade)	43.3 ml

Preparation: Silver staining

i) Fixation solution

Prepare immediately before fixation. Mix per sample:

10× Fixation Additive (cat# 4254-200-05)	10 µl
dH_2O	30 µl
Methanol	50 µl
Glacial acetic acid	10 µl

ii) 2× Staining Reagent #4 (cat# 4254-200-04)

Before first use, add 12 ml of dH_2O to bottle, stir until dissolved, and store at 4°C. Before each use, warm to room temperature.

iii) Staining solution (prepare immediately before staining)

The Staining Reagents #1, #2 and #3 are ready to use in the staining solution as described below:

For one sample, mix in an eppendorf tube:

dH_2O	35 µl
20× Staining Reagent #1 (cat# 4254-200-01)	5 µl
20× Staining Reagent #2 (cat# 4254-200-02)	5 µl
20× Staining Reagent #3 (cat# 4254-200-03)	5 µl

Mix well by tapping tube and add 50 µl 2× Staining Reagent #4.

For 10 samples:

dH_2O	350 µl
20× Staining Reagent #1 (cat# 4254-200-01)	50 µl
20× Staining Reagent #2 (cat# 4254-200-02)	50 µl
20× Staining Reagent #3 (cat# 4254-200-03)	50 µl

Mix by tapping tube and add 500 µl 2× Staining Reagent #4.

iv) Stop solution

Prepare a 5% acetic acid solution. 100 µl per sample area is required.

Tissue preparation

Cell samples should be prepared immediately before starting the assay, although success has been obtained using cryopreserved cells (see below). Cell samples should be handled under dimmed or yellow light to prevent DNA damage from ultraviolet light.

Buffers should be cooled to 4°C to inhibit endogenous damage occurring during sample preparation and to inhibit repair in cells. PBS must be calcium and magnesium free to inhibit endonuclease activities. The appropriate controls should also be included (see below).

Optimal results in the CometAssay® are usually obtained with 500-1000 cells per Comet-Slide™ sample area. Using 50 µl of a cell suspension at 1×10^5 cells per ml combined with 500 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 50 µl per sample.

i) Suspension Cells

Cell suspensions are harvested by centrifugation. Suspend cells at 1×10^5 cells/ml in ice cold 1× PBS (Ca^{++} and Mg^{++} free). Media used for cell culture can reduce the adhesion of LMAgarose to the CometSlide™.

ii) Adherent Cells

Gently detach cells from flask surface. Transfer cells and medium to centrifuge tube, perform cell count, and then pellet cells. Wash once in ice cold 1× PBS (Ca^{++} and Mg^{++} free). Suspend cells at 1 × 10^5 cells/ml in ice cold 1× PBS (Ca^{++} and Mg^{++} free). If high level of damage is seen in healthy population, reduce cell exposure to Trypsin or try alternative detachment methods such as scraping using a rubber policeman.

iii) Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA)

Wash the monolayer of cells with sterile PBS, warmed to 37°C. Add minimal amount of Trypsin- EDTA to coat the entire monolayer. Incubate flask at 37°C for 2 minutes or when cells easily detach upon tapping of flask. Add 10 ml of complete media (containing fetal bovine serum) to inactivate trypsin. Transfer cells and medium to centrifuge tube, perform cell count, and pellet cells (200×g). Wash once in ice cold 1× PBS (Ca^{++} and Mg^{++} free). Suspend cells at 1 × 10^5 cells/ml in ice cold 1× PBS (Ca^{++} and Mg^{++} free).

iv) Tissue Preparation

Place a small piece of tissue into 1-2 ml of ice cold 1× PBS (Ca^{++} and Mg^{++} free), 20 mM EDTA. Using small dissecting scissors mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet by centrifugation, and suspend at 1 × 10^5 cells/ml in ice cold 1× PBS (Ca^{2+} and Mg^{2+} free).

For blood rich organs (e.g., liver, spleen), chop tissue into large pieces (1-2mm^3), let settle for 5 minutes then aspirate and discard medium. Add 1-2 ml of ice cold 20 mM EDTA in 1× PBS (Ca^{++} and Mg^{++} free), mince the tissue into very small

pieces and let it stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet, and suspend at 1 $\times 10^5$ cells/ml in ice cold 1× PBS (Ca^{++} and Mg^{++} free).

v) Controls

A sample of untreated cells should always be processed as a control to account for assay variability, endogenous levels of damage within cells, and for additional damage that may occur during sample preparation. Control cells and treated cells should be handled in an identical manner. If UV damage is being studied; the cells should be kept in low level yellow light during processing.

Note: To generate positive samples for comet tails, treat cells with 100 µM hydrogen peroxide or 25 µM $KMnO_4$ for 20 minutes at 4°C. Treatment will generate significant oxidative damage in the majority of cells, thereby providing a positive control for each step in the alkaline comet assay.

vi) Cryopreservation of Cells Prior to CometAssay™

Certain cells, e.g. lymphocytes, may be successfully cryopreserved prior to performing the CometAssay™ (Visvardis et al, 1997). A pilot study should be performed to determine if cryopreservation is appropriate for the cells in use.

1. Centrifuge cells at 200 × g for 5 minutes.

2. Suspend cell pellet at 3×10^5 cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.

3. Transfer 50 µl aliquots into freezing vials.

4. Freeze at -70°C, with -1°C per minute freezing rate, overnight.

5. Transfer to liquid nitrogen for long term storage.

6. Recover cells by submerging in 37°C water bath until the last trace of ice has melted.

7. Add 500 µl ice cold 1× PBS (Ca^{++} and Mg^{++} free) to tube.

8. Centrifuge at 200 x g for 10 minutes at 4°C.

9. Suspend in 100 µl ice cold 1× PBS (Ca^{++} and Mg^{++} free) at ~1×10^5 cells/ml and proceed with CometAssay®.

Method: Neutral Comet Assay

1. Prepare Lysis Solution (see Preparation: Reagents for All Molecular Assays above) and cool at 4°C for at least 20 minutes before use.

2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened, and then cool in a 37°C water bath for at least 20 minutes.

3. Combine cells at 1 x 10^5/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl onto CometSlide™. Use side of pipette tip to spread agarose/cells over sample area.

Comet LMAgarose (molten and at 37°C from step 2)	100 µl
Cells in 1X PBS (Ca++ and Mg++ free) at 1 × 10^5/ml	10 µl

   Note: If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.

4. Place slides flat at 4°C in the dark (e.g. place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide™ area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.

5. Immerse slides in 4°C (Step 1) Lysis Solution for 1 hour or overnight for added sensitivity.

6. Remove slides from Lysis Buffer, drain excess buffer from slide and gently immerse in 50 ml of 4°C 1× Neutral Electrophoresis Buffer for 30 minutes.

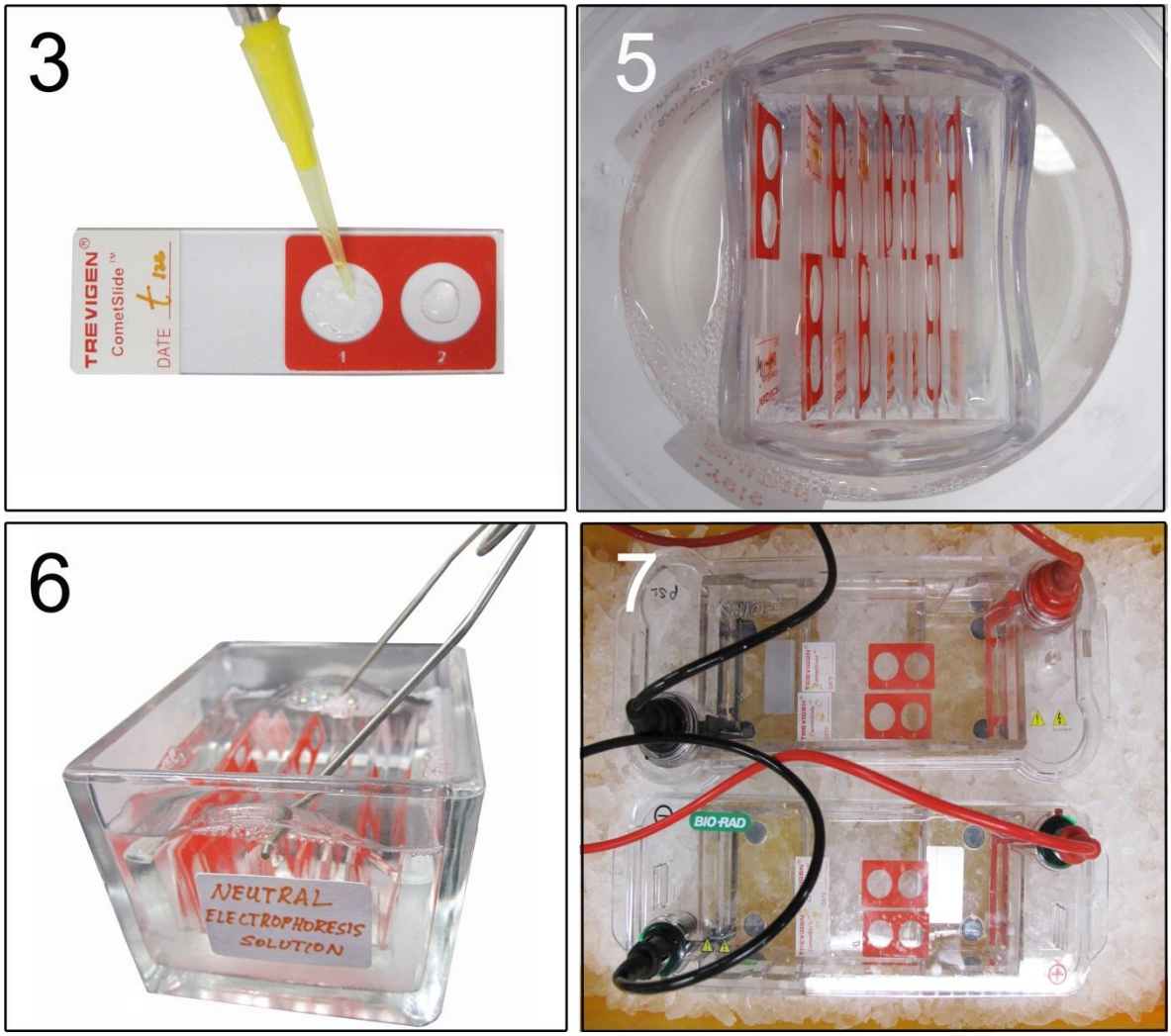
7. Add sufficient 4°C 1× Neutral Electrophoresis Buffer (not to exceed 0.5 cm above slides) into the electrophoresis tanks, place slides into the electrophoresis tanks (slide label adjacent to black cathode). Set power supply to 21 volts and apply voltage for 1 hour at 4°C. Voltage can be adjusted to 1volt per cm between electrodes for larger electrophoresis units.

8. Drain excess Neutral Electrophoresis Buffer and immerse slides in DNA Precipitation Solution for 30 minutes at room temperature.

9. Immerse slides in 70% ethanol for 30 minutes at room temperature.

10. Dry samples at 37°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.

11. Proceed to Silver Staining.



The above pictures illustrate procedures involved in steps 3 and 5-7 of the Neutral Comet Assay on pages 89-90.

Method: Alkaline Comet Assay

1. Prepare Lysis Solution (see Preparation: Reagents for All Molecular Assays above) and cool at 4°C for at least 20 minutes before use.

2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened. Place bottle in a 37°C water bath for at least 20 minutes to cool. The temperature of the agarose is critical or the cells may undergo heat shock.

3. Combine cells at 1 × 10^5/ml with molten LMAgarose (at 37°C) at a ratio of 1: 10 (v/v) and immediately pipette 50 µl onto CometSlide™. If necessary, use side of pipette tip to spread agarose/cells over sample area to ensure complete coverage of the sample area. If sample is not spreading evenly on the slide, warm the slide at 37°C before application. When working with many samples aliquot agarose into 37°C warmed tubes, add cells, mix gently by inversion, and spread 50µl onto sample area.

Comet LMAgarose (molten and at 37°C from step 2)	100 µl
Cells in 1× PBS (Ca^{++} and Mg^{++} free) at 1 x 10^5/ml	10 µl

4. Place slide flat at 4°C in the dark (e.g. place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide™ area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.

5. Immerse slide in 4°C Lysis Solution for 30 to 60 minutes. For added sensitivity or convenience incubate overnight at 4°C.

6. Drain excess buffer from slides and immerse in freshly prepared Alkaline Unwinding Solution, pH>13. WEAR GLOVES WHEN PREPARING OR HANDLING THIS SOLUTION.

7. Immerse CometSlide™ in Alkali Unwinding Solution for 20 minutes at room temperature or 1 hour at 4°C, in the dark.

8. Add sufficient 4°C Alkaline Electrophoresis Solution into the electrophoresis tanks, place slides into the electrophoresis tank (slide label adjacent to black cathode. Set power supply to 21 volts and apply voltage for 30 minutes. Voltage can be adjusted to 1volt per cm between electrodes for larger electrophoresis units.

9. Drain excess electrophoresis solution from slides and gently immerse twice in dH_2O for 5 minutes each, then in 70% ethanol for 5 minutes. Do not pour liquid over slides.

10. Dry samples at 37°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.

11. Proceed to Silver Staining.

Method: Silver staining

1. Cover the sample area with 100 µl of Fixation solution.

2. Incubate for 20 minutes at room temperature.

3. Rinse in dH_2O for 30 minutes. (Removal of all residual acetic acid is essential).

4. Cover sample area with 100 µl of Staining Solution.

5. Incubate at room temperature for 5 to 20 minutes (Intensity of staining can be visualized under the microscope using 10× objective, and reaction stopped when comets are easily visible).

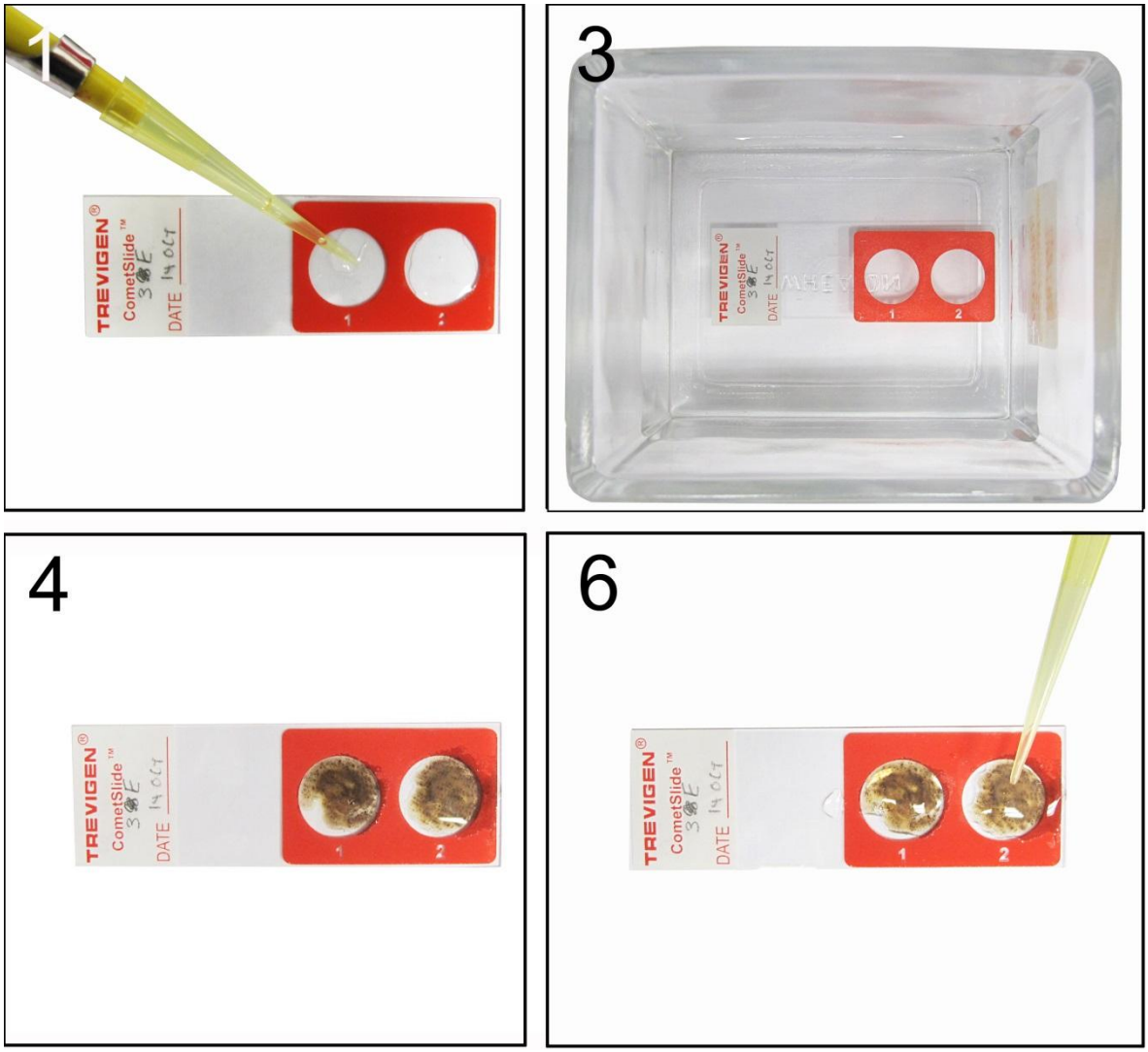
6. Stop reaction by covering samples with 100 µl of 5% acetic acid.

7. Incubate for 15 minutes.

8. Rinse in dH_2O.

9. Air dry and store in the dark.

The final Silver Staining solution is considered hazardous material. Disposal should be performed according to local and state regulations. It is recommended to tap solution off the slide into a container for safe disposal.



The above pictures illustrate procedures involved in steps 1, 3, 4 and 6 of the Silver Staining on page 94.

Silver Staining of DNA generates a brown to black stains easily detectable by microscopy. In healthy cells, the stain is confined to the nucleoid (comprised of high molecular weight DNA): undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleoid under the influence of an electric current. Whereas in cells that have accrued DNA damage, migrating fragments (comet tail) from the nucleoid (comet head) are observed. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. Common descriptors of DNA damage for alkaline comet assays are Percent DNA in the Tail, and Tail Moment. Percent DNA in the Tail is a normalized measure of the percent of total cell DNA found in the tail. Tail Moment is a damage measure combining the amount of DNA in the tail with distance of migration. In neutral comet assays, Tail Moment is primarily used, since tail length continues to increase in contrast to alkaline comet tails which have finite lengths.

Analyses of Molecular Assays

i) Qualitative Analysis (Alkaline CometAssay®)

The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for a healthy cell. Scoring can then be made according to nominal, medium or high intensity tail DNA content. At least 50 cells should be scored per sample.

ii) Quantitative Analysis (Alkaline and Neutral CometAssay®)

There are several image analysis systems that are suitable for quantitation of CometAssay® data. The more sophisticated systems include the microscope, camera and computer analysis package. These systems can be set up to measure

the length of DNA migration, image length, nuclear size, and calculate DNA damage parameters. At least 50 randomly selected cells should be analyzed per sample.

Conclusions

The biomarker techniques in this handbook explore various biological hierarchical functions: physiological/behavioural, cellular, biochemical, and molecular/genetic to assess organismal health as an indicator of ecological and habitat integrity. As compared to chemical analysis of environmental contaminants, these techniques offer the following benefits:

1) Rapid
2) Cheap
3) Easily deployable and repeatable
4) Measure integrated health function
5) Non-specific stress indicators (detects stress regardless of source)
6) Applicable to wide range of biomarker species
7) Ease of field data analysis and monthly/quarterly data logging maintenance. Possibility of transplantation manipulation of test organisms to gauge habitats where there are no background histories or data. For example: mussels with known background physiological responses to assays may be transplanted to test sites and assessed again after a month to identify environmental impacts.

It will be labour intensive and expensive to utilize the full biomarker battery (at least 8 – 12 indices) presented in this handbook, to verify potential effects of pollutants at tens or hundreds of sites along a sea coast. Hence, it is recommended that collaborative efforts be coordinated between interest groups, statutory boards and educational groups, for coordinated data collection over overlapping coastal sites as a “biological watch” for the coasts that will result in a continuous assessment of coastal environmental health. The data generated would be invaluable both scientifically as a chronological record of biological responses of target species over time, as well as an early warning signal that could alert management and policy of environmental impacts due to anthropogenic activities.

References

Aprikian, O., M. Levrat-Vernay, C. Besson, J. Busserolles, C. Remesy, C. and C. Demigne, 2001. Apple favourably affects parameters of cholesterol metabolism and of anti-oxidative protection in cholesterol-fed rats. Food Chemistry 75, 445– 452.

Bacon, G.S., B.A. MacDonald and J.E. Ward, 1998. Physiological responses of infaunal (*Mya arenaria*) and epifaunal (*Placopecten magellanicus*) bivalves to variations in the concentration and quality of suspended particles I. Feeding activity and selection. Journal of Experimental Marine Biology and Ecology 219, 105–125.

Barthel, D. and B. Wolfrath, 1989. Tissue sloughing in the sponge *Halichondria panicea*: a fouling organism prevents being fouled. Oecologia 78, 357-360

Bauman, J.W., J. Liu and C.D. Klaassen, 1993. Production of metallothionein and heat-shock proteins in response to metals. Fundamental & Applied Toxicology 21, 15-22.

Bayne, B.L., 1993. Feeding physiology of bivalves: time-dependent and compensation for changes in food availability. In: Dame, R.F. (Ed.), Bivalve Filter Feeders NATO ASI Series Sub-series G. Springer-Verlag, Berlin, Germany, pp. 1–24.

Bayne, B.L., 1998. The physiology of suspension feeding by bivalve molluscs: an introduction to the Plymouth "TROPHEE" workshop. Journal of Experimental Marine Biology and Ecology 219, 1–19.

Bayne, B.L., 1999. Physiological components of growth differences between individual oysters (*Crassostrea gigas*) and a comparison with *Saccostrea commercialis*. Physiological and Biochemical Zoology 72, 705–713.

Bayne, B.L., 2000. Relations between variable rates of growth, metabolic costs and growth efficiencies in individual Sydney rock oysters (*Saccostrea commercialis*). Journal of Experimental Marine Biology and Ecology 252, 185–203.

Bayne, B.L., J.I.P. Iglesias, A.J.S. Hawkins, E. Navarro, M. Heral and J.M. Deslouis-Paoli, 1993. Feeding behaviour of the mussel, *Mytilus edulis*: responses to variations in quantity and organic content of the seston. Journal of the Marine Biological Association of the U.K. 73, 813–829.

Benzie, I.F.F., W.Y. Chung and J.J Strain, 1999. ''Antioxidant'' (reducing) efficiency of ascorbate in plasma is not affected by concentration. Journal of Nutritional Biochemistry 10, 146–150.

Benzie, I.F.F. and J.J. Strain, 1996. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: the FRAP assay. Annals of Biochemistry 239, 70– 76.

Bergquist, P. R. 1978. Sponges. Hutchinson and Co. Ltd., London, United Kingdom.

Birmelin, C., R. K. Pipe, P. S. Goldfarb and D. R. Livingstone, 1999. Primary cell-culture of the digestive gland of the marine mussel *Mytilus edulis*: a time-course study of antioxidant- and biotransformation-enzyme activity and ultrastructural changes. Marine Biology 135, 65 - 75.

Boveris, A. and B. Chance, 1973. The mitochondrial generation of hydrogen peroxide. Biochemical Journal 134, 707–716.

Bricelj, V.M. and R.E. Malouf, 1984. Influence of algal and suspended sediment concentrations on the feeding physiology of the hard clam *Mercenaria mercenaria*. Marine Biology 84, 155–165.

Burlando, B., B. Marchi, I. Panfoli and A. Viarengo, 2002. Essential role of Ca^{2+}- dependent phospholipase A2 in estradiol-induced lysosome activation. American Journal of Physiology – Cell Physiology 283C, 1461–1468.

Cajaraville, M. P., I. Olabarrieta and I. Marigomez, 1996. *In vitro* activities in mussel hemocytes as biomarkers of environmental quality: a case study of Abra estuary (Biscay Bay). Ecology and Environmental Safety 35, 253–260.

Canesi, L., M. Betti, C. Ciacci, A. Scarpato, B. Citterio, C. Pruzzo and G. Gallo, 2004. Environmental estrogenes can affect the function of mussel hemocytes through rapid modulation of kinase pathways. General and Comparative Endocrinology 138, 58–69.

Chai, YC, S.S. Ashraf, K. Rokutan, R.B. Johnston Jr and J.A. Thomas, 1994. S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. Archives of Biochemistry and Biophysics 310, 273– 281.

Chiu, J.M.Y., T.Y.T. Ng, W.X. Wang, V. Thiyagarajan and P.Y. Qian, 2007. Latent effects of larval food limitation on filtration rate, carbon assimilation and growth in juvenile gastropod *Crepidula onyx*. Marine Ecology Progress Series 343, 173–182.

Cho, SM and W.G. Jeong, 2005. Spawning impact on lysosomal stability of the Pacific Oyster, *Crassostrea gigas*. Aquaculture, 244: 383-387.

Claisse, D., and C. Alzieu, 1993. Copper contamination as a result of antifouling paint regulations? Marine Pollution Bull. 26, 395-397

Clarke, M., 1999. The eVect of food availability on byssogenesis by the zebra mussel (*Dreissena polymorpha* Pallas). Journal of Molluscan Studies 65, 327–333

Clarke, M. and R.F. McMahon, 1996. Comparison of byssal attachment in dreissenid and mytilid mussels: mechanisms, morphology, secretion, biochemistry, mechanics and environmental influences. Malacological Review 29, 1–16.

Cole, J. A., S.R. Farley and R.K. Pipe, 1995. Alteration of the immune response of the common marine mussel *Mytilus edulis* resulting from exposure to cadmium. Diseases of Aquatic Organisms 22, 59–65.

Collins, A.R., and A. Azqueta, 2012. Single-cell gel electrophoresis combined with lesion-specific enzymes to measure oxidative damage to DNA, Methods in Cell Biology 112, 69–92.

Coughlan, J., 1969. The estimation of filtering rate from the clearance rate of suspensions. Marine Biology 2, 356-358.

Cranford, P.J., 2001. Evaluating the 'reliability' of filtration rate measurements in bivalves. Marine Ecology Progress Series 215, 303–306.

Cranford, P.J. and P.S. Hill, 1999. Seasonal variation in food utilization by the suspensionfeeding bivalve molluscs *Mytilus edulis* and *Placopecten magellanicus*. Marine Ecology Progress Series 190, 223–239.

Da Ros, L., F. Meneghetti and C. Nasci, 2002. Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy). Marine Environmental Research 54, 817-822.

Dayton, P. K. 1989. Interdecadal variation in an Antarctic sponge and its predators from oceanographic climate shifts. Science 245, 1484-1486.

Dennog, C., P. Radermacher, Y.A. Barnett and G. Speit, G., 1999. Antioxidant status in humans after exposure to hyperbaric oxygen. Mutatation Research 428, 83– 89.

Dikkeboom, R., J.M.G.H. Tijnagel, E.C. Mulder. and W.L.P.W. van der Knaap, 1987. Hemocytes of pond snail *Lymnaea stagnalis* generate reactive forms of oxygen. Journal of Invertebrate Pathology 49, 321–331.

Dissanayake, A., T.S. Galloway. and M.B. Jones, 2011. Seasonal differences in the physiology of *Carcinus maenas* (Crustacea: Decapoda) from estuaries with varying levels of anthropogenic contamination. Estuarine and Coastal Shelf Science, 93, 320 – 327.

Ens, S., A.B. Olson, C. Dudley, N.D. Ross III, A.A. Siddiqi, K.M. Umoh and M.A. Schneegurt, 2012. Inexpensive and Safe DNA Gel Electrophoresis Using Household Materials. Biochemistry and Molecular Biology Education 40(3), 198 – 203.

Erdogan, C., Y. Unlucerci, A. Turkmen, A. Kuru, O. Cetin, and S. Bekpinar, 2002. The evaluation of oxidative stress in patients with chronic renal failure. Clinica Chimica Acta, 322, 157– 161.

Farrell, E.D. and T.P. Crowe, 2007. The use of the byssus by *Mytilus edulis* as an active defence against *Nucella lapillus*. Journal of the Marine Biological Association of the United Kingdom, 87, 559–564

Ferreira, A. and H. Dolder, 2003. Cytochemical study of spermiogenesis and mature spermatozoa in the lizard *Tropidurus itambere* (Reptilia, Squamata). Acta Histochemica 105, 339–352.

Franzellitti, S., S. Buratti, F. Donnini and E. Fabbri, 2010. Exposure of mussels to a polluted environment: Insights into the stress syndrome development. Comparative Biochemistry and Physiology C 152(1), 24-33.

Galtsoff, P. S. 1925. Regeneration after dissociation (an experimental study on sponges). Journal of Experimental Zoology 42, 183-221.

Gili, J.M. and R. Coma, 1998. Benthic suspension feeders: their paramount role in littoral marine food webs. Trends in Ecology and Evolution 13, 316-321.

Gladstone, W. 1996. Trace metals in sediments, indicator organisms and traditional seafoods of the Torres Strait. Report series 5a. Townville, Australia. Great Barrier Reef Marine Park Authority.

Gladstone, W., and I.J. Dight, 1994. Torres Strait baseline study. Marine Pollution Bulletin 29, 121-125.

Goh, B., 2008. Research on Ecotoxicology and Applications in Singapore: Description of the Sponge Aggregation Assay. In: Y. Murakami, K. Nakayama, S.-I. Kitamura, H. Iwata and S. Tanabe (Ed.), Interdisciplinary Studies on Environmental Chemistry – Biological Responses to Chemical Pollutants. Tokyo: Terra Scientific Publishing Company (Terrapub), pp. 17 – 29.

Goh, B.P.L. and C.H. Lai, 2014. Establishing the thermal threshold of the tropical mussel, *Perna viridis* in the face of global warming. Marine Pollution Bulletin 85, 325-331.

Goering, P.L. 1993. Lead-protein interactions as a basis for lead toxicity. Neurotoxicology 14, 45-60.

Gorbushin, A.M. and N.V. Iakovleva, 2007. Functional characterization of *Littorina littorea* (Gastropoda: Prosobranchia) blood cells. Journal of Marine Biology Association of the United Kingdom 87, 741–746.

Grace, A. L., and L.F. Grainey Jr., 1987. The effects of copper on the heart rate and filtration rate of *Mytilus edulis.* Marine Pollution Bulletin, 18(2), 87-91.

Griffliths, C.L. amd J.A. King, 1979. Energy expended on growth and gonadal output in the ribbed mussel *Aulacomya ater*. Marine Biology 53, 217– 222.

Guidi, P., G. Frenzilli, M. Benedetti, M. Bernardeschi, A. Falleni, D. Fattorini, F. Regoli, V. Scarcelli and M. Nigro, 2010. Antioxidant, genotoxic and lysosomal biomarkers in the freshwater bivalve (*Unio pictorum*) transplanted in a metal polluted river basin. Aquatic Toxicology 100(1), 75-83.

Halliwell, B. and J.M.C. Gutteridge, 1999. Free Radicals in Biology and Medicine, 3rd ed. Oxford University Press.

Halvorsen, B.L., K. Holte, C.W. Mari, I. Barikmo, E. Hvattum, S.F. Remberg, A. Wold, K. Haffner, H. Baugerød, L.F. Andersen, J.O. Moskaug, D.R. Jacobs and R. Blomhoff, 2002. A systematic screening of total antioxidants in dietary plants. Journal of Nutrition 132, 461–471.

Hansen, I.V., J.M. Weeks, and H. Depledge, 1995. Accumulation of copper, zinc, cadmium and chromium by the marine sponge *Halichondria panicea* Pallas and implications for biomonitoring. Marine Pollution Bulletin 31, 133-138.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature 381, 571-80.

Hauton, C, L.E. Hawkins and S. Hutchinson, 1998. The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster *Ostrea edulis* (L). Comparative Biochemistry and Physiology B 119, 619-623.

Hauton, C, L.E. Hawkins and S. Hutchinson, 2001. Response of haemocyte lysosomes to bacterial inoculation in the oysters *Ostrea edulis* L. and *Crassostrea gigas* (Thunberg) and the scallop *Pecten maximus* (L.). Fish and Shellfish Immunology 11, 143-153.

Hawkins, A.J.S. and B.L. Bayne, 1985. Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. Marine Ecology Progress Series 25, 181 –188.

Hawkins, A.S.J., M.R. James, R.W. Hickman, S. Hatton and M. Weatherland, 1999. Modeling of suspension feeding and growth in the green-lipped mussel *Perna canaliculus* exposed to natural and experimental variations of seston availability in the Marlborough Sounds, New Zealand. Marine Ecology Progress Series 191, 217–232

Hooper, J.N.A. and R.W.M. Soest, 2002. Systema Porifera: a guide to the classification of sponges. Kluwer Academic/Plenum Publishers, New York, NY.

Jorgensen, C.B., 1990. Bivalve Filter Feeding: Hydrodynamics, Bioenergetics, Physiology and Ecology. Olsen and Olsen, Denmark.

Jorgensen, C.B., 1996. Bivalve filter feeding revisited. Marine Ecology Progress Series 142, 287–302.

Jorgensen, C.B., P. Famme, H.S. Kristensen, P.S. Larsen, F. Mohlenberg and H.U. Riisgard, 1986. The bivalve pump. Marine Ecology Progress Series 34, 69–77.

Jorgensen, C.B., P.S. Larsen, F. Mohlenberg and H.U.Riisgard, 1988. The mussel pump: properties and modeling. Marine Ecology Progress Series 45, 205–216.

Jorgensen, C.B., P.S. Larsen and H.U. Riisgard, 1990. Effect of temperature on the mussel pump. Pubblicazioni della Stazione Zoologica di Napoli. (I: Marine Ecology) 64, 69–71.

Koehler, A., K. Soeffker and E. Wahl, 2002. Functional and morphological changes of lysosomes as prognostic biomarker of toxic injury in a marine flatfish *Platichthys flesus* (L.). Environmental Toxicology and Chemistry 21, 2434–2444

Kooijman, S.A.L.M., 2010. Dynamic Energy Budget Theory for Metabolic Organisation. Cambridge University Press. 514 pp.

Kultz, D. 1996.. Plasticity and stressor specificity of osmotic and heat-shock responses of *Gillichthys mirabilis* gill cells. American Journal of Physiology. Cell Physiology C 40, 1181-1193.

Lachance, A.A., B. Myrand, R. Tremblay, V. Koutitonsky and E.Carrington, 2008. Biotic and abiotic factors influencing attachment strength of blue mussels *Mytilus edulis* in suspended culture. Aquatic Biology 2, 119–129.

Lee, A., D.I. Thurman and M. Chopra, 2000. Consumption of tomato products with olive oil but not sunflower oil increases the antioxidant activity of plasma, Free Radical Biology and Medicine 29(10), 1051 – 1055.

Leung, K. M. Y. and R. W. Furness, 2001. Survival, growth, metallothionein and glycogen levels of *Nucella lapillus* (L.) exposed to subchronic cadmium stress: the influence of nutritional state and prey type. Marine Environmental Research 52, 173-194.

Lewis, B.G., 1985. Mussel control and chlorination. Report No. TPRD/L/2810/R85. Central Electricity Research Laboratories, Leatherhead, Surrey, p. 33

Lowe, D.M., V.U. Fossato and M.H. Depledge, 1995a. Contaminant induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from Venice Lagoon: an *in vitro* study. Marine Ecology Progress Series 129, 189–196.

Lowe, D.M., M.N. Moore and B.M. Evans, 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Marine Ecology Progress Series 91, 135-140.

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

Luzio, J.P., P.R. Pryor and N.A. Bright, 2007. Lysosomes: fusion and function. Nature Reviews Molecular Cell Biology 8(8), 622-632.

Mamaca, E., R.K. Bechmann, S. Torgrimsen, E. Aas. A. Bjørnstad, A. Baussant, T. Baussant and S. LeFloch, 2005. The neutral red lysosomal retention assay and comet assay on haemolymph cells from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene. Aquatic Toxicology 75, 191-201.

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

Martinez-Gomez, C, J. Benedicto, J.A. Campillo and M. Moore, 2008. Application and evaluation of the neutral red retention (NRR) assay for lysosomal stability in mussel populations along the Iberian Mediterranean coast. Journal of Environmental Monitoring 10(4), 490-499.

Meadows, P. S. and P. Shand, 1989. Experimental analysis of byssus thread production by *Mytilus edulis* and *Modiolus modiolus* in sediments. Marine Biology 101(2), 219–226.

Mestril, R., S.-H. Chi, R. Sayen and W.H. Dillman, 1994. Isolation of a novel inducible rat heat-shock protein HSP70. gene and its expression during ischemiarhypoxia and heat shock. Biochemical Journal 298, 561-9.

Moore, M.N., 1980. Cytochemical determination of cellular responses to environmental stressors in marine organisms. Rapports et Proces-verbaux des Réunions. Conseil International pour l'Éxploration de la Mer 170, 7–15.

Moore, M.N., A. Kohler, D. Lowe and A. Viarengo, 2008. Lysosomes and autophagy in aquatic animals. Autophagy, 451: 581-620.

Moore, M.N., 1976. Cytochemical demonstration of latency of lysosomal hydrolases in the digestive cells of the common mussel, *Mytilus edulis*, and changes induced by thermal stress. Cell and Tissue Research 175, 279–287.

Morris, D. L., 1948. Quantitative Determination of Carbohydrates With Dreywood's Anthrone Reagent. Science 107, 254.

Müller, W.E.G., J. Arendes, B. Kurelec, R.K. Zahn and I. Muller, 1977. Species-specific aggregation factor in sponges. Sialyltransferase associated with aggregation factor. Journal of Biological Chemistry 271, 23558-23565.

Myrmel, T., J.D. McCully, L. Malkin, I.B. Krukenkamp and S. Levitsky, 1994. Heat shock protein 70 mRNA is induced by anaerobic metabolism in rat hearts. Circulation 90, 299-305.

Nepple, B.B. and R. Bachofen, 1997. Induction of stress proteins in the phototrophic bacterium *Rhodobacter sphaeroides*. Federation of European Microbiological Societies (FEMS) Microbiology Letters 153, 173-80.

Niemeyer, H.B. and M. Metzler, 2003. Differences in the antioxidant activity of plant and mammalian lignans. Journal of Food Engineering 56, 255–256.

Ohkuma, S, Y. Moriyama and T. Takano, 1982. Identification and characterization of a proton pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. Proceedings of the National Academy of Science, USA 79, 2758-2762.

Olesen, T.M., and J.M. Weeks, 1994. Accumulation of Cd by the marine sponge *Halichondria panicea* Pallas: effects upon filtration rate and its relevance for biomonitoring. Bulletin of Environmental Contamination and Toxicology 52(5), 722-728.

Palais, F., C. Mouneyrac, O. Dedourge-Geffard, L. Giambérini, S. Biagianti and A. Geffard, 2011. One-year monitoring of reproductive and energy reserve cycles in transplanted zebra mussels (Dreissena polymorpha). Chemosphere 83(8), 1062-1073.

Pechenik, J.A., J.N. Jarrett and J. Rooney, 2002. Relationships between larval nutritional experience, larval growth rates, juvenile growth rates, and juvenile feeding rates in the prosobranch gastropod *Crepidula fornicata*. Journal of Experimental Marine Biology and Ecology 280, 63–78.

Peña, M.M.O., L. Jaekwon, and D.J. Thiele, 1999. A delicate balance: homeostatic control of copper uptake and distribution. Journal of Nutrition 129, 1251–1260.

Philp, R.B., 1997. Effects of pH and oxidant stressors (hydrogen peroxide and bleach) on calcium-induced aggregation of cells of the marine sponge *Microciona prolifera*. Comparative Biochemistry and Physiology C 118, 347–351.

Pipe, R.K., 1993. The generation of reactive oxygen metabolites by the haemocytes of the mussel *Mytilus edulis*. Developmental and Comparative Immunology 16, 111–122.

Pizzichini, M., M. Fonzi, L. Sugherini, L. Fonzi, A. Gasparoni, M. Comporti and A. Pompella, 2002. Release of mercury from dental amalgam and its influence on salivary antioxidant activity. Science of the Total Environment 284, 19–25.

Pörtner, H.O., 2002. Climate change and temperature dependent biogeography: systemic to molecular hierarchies of thermal tolerance in animals. Comparative Biochemistry and Physiology A 132, 739–761.

Price, H. A., 1980. Seasonal variation in the strength of byssal attachment of the common mussel *Mytilus edulis* L. Journal of the Marine Biological Association of the United Kingdom 60, 1035–1037.

Price, H.A., 1982. An analysis of factors determining seasonal variation in the byssal attachment strength of *Mytilus edulis*. Journal of the Marine Biological Association of the United Kingdom 62, 147–155

Prior, R.L. and G. Cao, 1999. *In vivo* total antioxidant capacity: comparison of different analytical methods. Free Radical Biology and Medicine 27 (11/12), 1173–1181.

Reish, D.J., P.S. Oshida, F.G. Wilkes, A.J. Mearns, T.C. Ginn and R.S. Carr, 1984. Effects on saltwater organisms. Journal of the Water Pollution Control Federation 56, 759-774.

Reiswig, H.M., 1971. Particle feeding in natural populations of three marine demosponges. Biological Bulletin 141, 568-591.

Riisgard, H.U., 2001a. Physiological regulation versus autonomous filtration in filter feeding bivalves: starting points for progress. Ophelia 54, 193–209.

Riisgard, H.U., 2001b. On measurement of filtration rates in bivalves — the stony road to reliable data: review and interpretation. Marine Ecology Progress Series 211, 275–291.

Riisgard, H.U., C. Kittner and D.F. Seerup, 2003. Regulation of opening state and filtration in filter-feeding bivalves (*Cardium edule, Mytilus edulis, Mya arenaria*) in response to low algal concentration. Journal of Experimental Marine Biology and Ecology 284, 105–127.

Riisgard, H.U., and P.S. Larsen, 2007. Viscosity of seawater controls beat frequency of water-pumping cilia and filtration rate of mussels *Mytilus edulis*. Marine Ecology Progress Series 343, 141–150.

Riisgard, H.U., S. Thomassen, H. Jakobsen, J.M. Weeks and P.S. Larsen, 1993. Suspension-feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus* - effects of temperature on filtration-rate and energy cost of pumping. Marine Ecology Progress Series 96, 177-188.

Ritossa, F.M., 1962. A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia 18, 571-573.

Sanders, B.M., 1990. Stress-proteins: Potential as multitiered biomarkers. In L. Shugart and J. McCarthy (Ed.) Environmental Biomarkers. Boca Raton, Florida: Lewis Publishers, pp. 165-91.

Sanders, B.M., L.S. Martin, W.G. Nelson, D.K. Phelps and W. Welch, 1991. Relationships between accumulation of a 60 kDa stress protein and scope for growth in *Mytilus edulis* exposed to a range of copper concentrations. Marine Environmental Research 31, 81-97.

Sattler, L and F.W. Zerban, 1948. The Dreywood anthrone reaction as affected by carbohydrate structure. Science, 108, 207.

Seglen, P.O., 1983. lnhibitors of lysosomal function. Methods in Enzymology 96, 737-764.

Seifter, S., S. Dayton, B. Novic and E. Muntwyler, 1950. The estimation of glycogen with the antrone reagent. Archives of Biochemistry 25, 191–200.

Simpson, T.L., 1984. The Cell Biology of Sponges. Springer-Verlag, New York, NY

Singh, N.P., M.T. McCoy, R.R. Tice and E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184–191.

Sloff, W., D. de Zwart and J.M Marquenie, 1983. Detection limits of a biological monitoring system for chemical water pollution based on mussel activity. Bulletin of Environmental Contamination and Toxicology 30, 400-405.

Sohal, R.S. and R. Weindruch, 1996. Oxidative stress, caloric restriction and aging. Science 273, 59–63.

Sommers, C.H., X. Fan, P. Handel and K.B. Sokorai, 2003. Effect of citric acid on the radiation resistance of *Listeria monocytogenes* and frankfurter quality factors. Meat Science 63, 407– 415.

Tsai, P., J. McIntosh, P. Pearce, B. Camden and B.R. Jordan, 2002. Anthocyanin and antioxidant capacity in Roselle (*Hibiscus sabdariffa* L.) extract. Food Research International 35, 351– 356.

Visvardis, E.E., A.M. Tassiou and S.M. Piperakis, 1997. Study of DNA damage induction and repair capacity of fresh cryopreserved lymphocytes exposed to H_2O_2 and γ-irradiation with the alkaline comet assay. Mutation Research 383, 71–80.

Vogal, S., 1977. Current induced flow through living sponges in nature. Proceedings of the National Academy of Sciences USA 74, 2069-2071.

Wang, Y. J., M. Hua, W.H. Wong, P.K.S. Shin and S.G. Cheung, 2011. The combined effects of oxygen availability and salinity on physiological responses and scope for growth in the green-lipped mussel *Perna viridis*. Marine Pollution Bulletin 63(5-12), 255-261.

Ward, J.E. and B.A. MacDonald, 1996. Pre-ingestive feeding behaviours of two sub-tropical bivalves (*Pinctada imbricata* and *Arca zebra*): Responses to an acute increase in suspended sediment concentration. Bulletin of Marine Science 59, 417–432.

Watling, H.. 1981. The effects of metals on mollusc filtering rates. Transactions of the Royal Society of South Africa 44(3), 441-451.

Webster, N.S., R.I. Webb, M.J. Ridd, R.S. Hill and A.P. Negri, 2001. The effects of copper on the microbial community of a coral reef sponge. Environmental Microbiology 3, 19-31.

Widdows, J., 2001. Bivalve clearance rates: inaccurate measurements or inaccurate reviews and misrepresentation? Marine Ecology Progress Series 221, 303–305.

Williams, J.H., A.M. Farag, M.A. Stansbury, P.A. Young, H.L. Bergman and N.S. Petersen, 1996. Accumulation of HSP70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. Environmental Toxicology and Chemistry 15, 1324-1328.

Winkle, W.V.J.. 1970. Effect of environment factors on byssal thread formation. Marine Biology 7, 143 –148.

Yonge, C.M., 1962. On the primitive significance of the byssus in the Bivalvia and its effects in evolution. Journal of the Marine Biological Association of the United Kingdom 42, 113–125.

Young, G.A., 1985. Byssus-thread formation by the mussel *Mytilus edulis*: effects of environmental factors. Marine Ecology Progress Series 24, 261–271.

Zardi G.I., C.D. McQuaid and K.R. Nicastro, 2007. Balancing survival and reproduction: seasonality of wave action, attachment strength and reproductive output in indigenous *Perna perna* and invasive *Mytilus galloprovincialis* mussels. Marine Ecology Progress Series 334, 155–163.

Zhang, Z., X. Li, M. Vandepeer and W. Zhao, 2006. Effects of water temperature and air exposure on the lysosomal membrane stability of hemocytes in Pacific oysters, *Crassostrea gigas* (Thunberg). Aquaculture 256, 502–509.