ICES TECHNIQUES IN MARINE ENVIRONMENTAL SCIENCES

NO. 40

SEPTEMBER 2006

BIOLOGICAL EFFECTS OF CONTAMINANTS: MEASUREMENT OF SCOPE FOR GROWTH IN MUSSELS

JOHN WIDDOWS AND FRED STAFF

PLYMOUTH MARINE LABORATORY
PROSPECT PLACE, THE HOE
PLYMOUTH, PL1 3DH
UNITED KINGDOM

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

ICES Techniques in Marine Environmental Sciences is issued by the International Council for the Exploration of the Sea. This series presents detailed descriptions of methods and procedures relating to chemical and biological measurements in the marine environment. Most techniques described have been selected for documentation based on performance in ICES or other intercalibration or intercomparison exercises: they have been carefully evaluated and shown to yield good results when correctly applied. They have also been subject to review by relevant ICES working groups, but this is not to be construed as constituting official recommendation by the Council.

Correspondence

Correspondence and inquiries of a general nature should be directed to the General Secretary. Correspondence concerning the details of any method or procedure should be directed to the author(s) of the publication in question.

Subscriptions and orders

Standing subscriptions and orders for individual numbers may be placed with the ICES Secretariat by post, Telefax, or e-mail. Further information about ICES publications, including ordering and payment by credit card, cheque, and bank transfer, can be found at http://www.ices.dk under "Products". Numbers are issued at irregular intervals.

ISBN [redacted] 978-87-7482-292-9 https://doi.org/10.17895/ices.pub.5064

ISSN [redacted] 2707-6997

Contents

Biological effects of contaminants: Measurement of scope for growth in mussels

John Widdows and Fred Staff

ICES. 2006. Biological effects of contaminants: Measurement of scope for growth in mussels. By J. Widdows and F. Staff. ICES Techniques in Marine Environmental Sciences. No. 40. 30 pp.

Abstract

Scope for growth (SFG) is a method of assessing the whole-animal physiological response to sublethal stress induced by pollutants. It has been applied widely in small- and large-scale pollution monitoring programmes in various regions of the world, ranging from temperate to tropical. SFG was primarily developed for use with suspension-feeding mussels (*Mytilus edulis* or similar indigenous species) and in combination with the analysis of chemical contaminants in mussel tissues. SFG is based on the measurement of physiological responses, such as feeding and respiration rate, and is derived from the difference between energy acquisition (rate of feeding and digestion) and energy expenditure (metabolic rate). The method has been successfully tested nationally in a range of UK monitoring programmes and internationally as part of IOC (Intergovernmental Oceanographic Commission) Biological Effects Workshops to evaluate and compare pollution effects measurements at different levels of biological organization.

Keywords

Scope for growth (SFG), mussels, *Mytilus edulis*, sublethal stress response, pollution monitoring.

1 Introduction

1.1 The concept of scope for growth

Growth provides one of the most sensitive measures of stress in an organism because growth integrates major physiological responses, specifically the balance between processes of energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion). Each physiological response can be readily determined in bivalves, converted into energy equivalents (J h^{-1}) and alterations in the energy available for growth and reproduction (scope for growth), and can be quantified using the balanced energy equation:

$$C - F = A = R + E + P$$

or

$$P = A - (R + E)$$

where

C = total consumption of food energy;
F = faecal energy loss;
A = absorbed food energy;
R = respiratory energy expenditure;
E = energy lost as excreta;
P = energy available for growth and reproduction (scope for growth).

In many laboratory and field studies conducted before 1990, all components of the energy equation were routinely measured. However, these studies consistently showed that energy lost via excreta was a small proportion (<5%) of the total energy budget (Widdows, 1993). Therefore for routine monitoring, the balanced energy equation can be further simplified to:

$$SFG = A - R$$

Scope for growth (SFG) provides an instantaneous measure of the energy status of an animal, which can range from maximum positive values under optimal conditions, declining to negative values when an animal is severely stressed and utilizing body reserves. Although direct measurements of total production and growth rate are often difficult to quantify and interpret in relation to pollution (Widdows and Donkin, 1992), SFG is rapidly determined, providing a sensitive, quantitative, and integrated response that can be related to the contaminant levels in the body tissues.

This approach, based on the combined measurement of SFG and chemical contaminants in mussels, has been used successfully to detect, quantify, and identify the potential causes of pollution in estuaries and bays (typically over small spatial scales of ca. 10 km; reviewed by Widdows and Donkin, 1992), as well as over larger spatial scales of >1000 km of North Sea coastline (Widdows *et al.*, 1995a) and the Irish Sea coastline (Widdows *et al.*, 2002). Furthermore, SFG has been applied over a wide range of latitudes from subtropical (Bermuda; Widdows *et al.*, 1990) to Subarctic (Iceland; Halldórsson *et al.*, 2005). Not only has SFG been correlated with concentrations of toxic contaminants in the tissues of mussels (Annex 1), but recent studies have also demonstrated that SFG correlates with measures of biodiversity in the benthic community (Crowe *et al.*, 2004). Therefore SFG can provide an effective indicator of pollution effects at the individual and at the community levels.

The methodology for determining the scope for growth of mussels (and other bivalve species) has been applied routinely to both toxicant-exposed mussels in laboratory studies and mussels collected from polluted environments. Laboratory studies are used to establish relationships between toxicant tissue concentrations and SFG (some of which are summarized in Annex 1), which can then be used to provide a quantitative toxicological interpretation of SFG and tissue

contaminant levels in field monitoring programmes (Widdows and Donkin, 1992; Widdows *et al.*, 1995a, 2002).

The underlying objective of both field and laboratory studies is to maintain and measure the SFG of individual mussels under "near optimal" conditions, so that the SFG will be maximized at a given ration level, and any reduction in SFG will reflect the stress induced by the toxicants accumulated in their body tissues. Although the SFG and actual growth of an animal depends on the food available, at higher food concentrations the ingestion rate, and therefore SFG, becomes relatively independent of ration through rejection mechanisms for excess food. This occurs in the form of increased production of pseudofaeces and faeces, leading to a reduction in food absorption efficiency with increasing food intake (Widdows, 1978a, b; Navarro and Widdows, 1997). Therefore, by standardizing the ration level in SFG measurements, the food absorption efficiency remains relatively constant, and food is removed as a key variable, so allowing SFG to reflect the underlying impact of the total toxicant load accumulated within the body tissues (Widdows and Johnson, 1988; Widdows *et al.*, 1990, 1995a, b, 1997, 2002). Although this SFG measurement does not predict the actual growth in the field, because food availability in the coastal environment is temporally and spatially variable and difficult to measure routinely, it does reflect the overall growth potential for individuals and mussel populations. For example, mussels from the Liverpool Bay and Morecombe Bay region of the Irish Sea had the lowest SFG values, and this was consistent with very low growth rates (an order of magnitude lower than unpolluted areas; Widdows *et al.*, 2002). Subsequent studies by Crowe *et al.* (2004) have demonstrated a lower biodiversity within the mussel bed community at the Irish Sea study sites with low mussel SFG.

In addition, more detailed chemical analyses of the mussel tissues have confirmed that the lowered SFG and biodiversity values were correlated with increased concentrations of hydrocarbons accumulated in the mussel tissues, particularly those associated with the "unresolved complex mixture" (Crowe *et al.*, 2004). Recent studies by Donkin *et al.* (2003) and A. Booth *et al.* (unpublished data) have begun to identify these previously unresolved compounds and shown them to be toxic to mussels. This demonstrates that SFG is able to detect and quantify pollution impact and that subsequent independent studies were able to analyse and identify the nature of the toxicants in more detail and show changes at the population and community level.

1.2 General description of the test organism: suspension feeding mussels

Mussels (*Mytilus edulis*), like most bivalves, are suspension feeders that can pump considerable volumes of water through their large gills and filter out small particulate food items from the water column. Mussels are particularly efficient filter-feeders, removing particles between ca. 3 µm and >50 µm in diameter with 100% efficiency (Møhlenberg and Riisgard, 1978). The total amount of particulate matter present in suspension (= seston) contains several food types that potentially can be utilized by mussels (Widdows *et al.*, 1979). The major utilizable component is phytoplankton, but bacteria and fine organic detritus are also important in supplementing the algal diet. Mussels undergo a seasonal cycle of somatic and shell growth (spring and summer), gametogenesis (autumn/winter), and spawning of gametes (late winter/spring). The eggs are fertilized externally in the water, where they develop into a free-swimming pelagic larval stage that feeds and grows in the water column for 2–3 weeks before settlement and then metamorphosis, marking the start of a sessile mode of life.

The preferred season for measuring SFG of field-collected mussels is during the period of maximum growth potential (i.e. from early summer to early autumn). It is important to avoid measurement of SFG or any other cellular/biochemical response during the spawning period, the timing of which is variable depending on latitude and seasonal temperature regime (but

generally late winter/spring). The process of sampling and transportation of mussels at this time increases the probability of inducing the release of gametes (particularly following a prolonged period of air exposure or a temperature/physical shock), and this naturally stresses the animal. The release of gametes makes it very difficult to perform SFG measurements successfully. In addition, it is advisable not to measure SFG in autumn during a period of natural quiescence before the onset of gametogenesis. At this time, the feeding and metabolic activities of mussels are at a minimum. Therefore, before undertaking any study using cellular or physiological responses on a new bivalve species, it is important to carry out some preliminary studies to establish the particular species requirements and the appropriate protocols. For example, a species may be sensitive to light or exhibit a diurnal cycle of activity (e.g. *Arca zebra*; Widdows *et al.*, 1990). However, in the case of *M. edulis*, there is no evidence of a significant diurnal cycle or sensitivity to light, but they do respond to sudden changes in light intensity (i.e. shading).

1.3 Source of animals

Mussels to be used in laboratory toxicity testing, and mussels chosen to represent clean reference sites in field studies, should be collected from a location that is free from significant chemical contamination (i.e. removed from local sewage inputs, urban development, and industry). Mussels collected from the mouth of most estuaries are not representative of a clean reference site. It is advisable to analyse body tissues for contaminants, particularly organics such as hydrocarbons, to confirm that the site is not significantly contaminated. (Visual assessment of the site is not sufficient.)

Mussels used for SFG can be collected either from native populations or from specific sites where mussels from a clean reference site have been transplanted and exposed in cages for a period of >4 w.

1.4 Laboratory vs. field measurement

Physiological energetic responses of mussels can be measured either in the field using a mobile laboratory (e.g. Widdows *et al.*, 1987, 1995b) or in the laboratory under standardized conditions (e.g. Widdows and Johnson, 1988; Widdows *et al.*, 1995a; Widdows *et al.*, 2002). Comparative methodological studies have found no significant difference between laboratory and field measurements of SFG (Widdows, 1983; Widdows *et al.*, 1995a). This is the case where laboratory measurements are made under standardized conditions in high quality seawater, after a brief period of recovery (ca. 24 h in flowing seawater) following transportation to the laboratory under cool (~5°C) air exposed conditions (for ca. 24 h), and before they are able to recover from pollution-induced stress. When the objective is primarily to quantify the decline in growth potential or the degree of stress induced by environmental pollution, then measurement under standardized laboratory conditions is recommended, based on convenience, cost, and efficiency. Under controlled laboratory conditions, natural environmental variables (such as food availability, temperature, salinity, and dissolved oxygen) are held constant, so that the physiological responses reflect the underlying effects of toxic contaminants accumulated in the tissues. The basic physiological responses of mussels (such as feeding and respiration rate) remain relatively independent of short-term changes in natural environmental variables over a wide range of conditions; for example food/seston concentration (0.1–20 mg seston l^{-1}; Widdows *et al.*, 1979; Kiørboe *et al.*, 1980), temperature (6–20°C; Widdows, 1976), and salinity (20–33; Widdows, 1985b). In addition, transplantation experiments over >1000 km have shown that any measurable differences in physiological responses and growth rates of different populations reflect environmental factors rather than genetic differences (Kautsky *et al.*, 1990; Widdows *et al.*, 1995a), permitting the direct comparison of mussels over a wide geographical area. This does not imply that genetically determined population differences in physiological responses do not exist, but that they are

only apparent under extreme environmental conditions (e.g. elevated temperatures and reduced salinities).

In field studies where mussels are collected from various sites to assess pollution effects, all measurements should be made at a standard temperature (e.g. mean ambient UK summer seawater temperature of 15°C), in air-saturated, high quality seawater at full salinity (~34), and feeding with high quality algal food (e.g. *Isochrysis galbana*, *Phaeodactylum tricornutum*, or *Tetraselmis suecica*, ideally in flow-through conditions at ~0.5 mg l^{-1}).

2 Material

The following material and equipment are required for SFG measurement:

1) Measure sixteen mussels (*M. edulis*) of a standard size (4 cm shell length). This sample size has the power to detect statistically and environmentally significant differences between populations/sites (Bayne *et al.*, 1981) and is a practical number of individuals to measure. Usually 25 specimens are collected and available for physiological measurements, with up to 200 mussels for analysis of chemical contaminants in body tissues.

2) Polystyrene insulated containers (inner core ~20 × 20 cm), each with two frozen cool packs (to transport mussels at a temperature of ~5°C) and disposable nappies (to absorb water and buffer mussels from cool packs).

3) Sufficient high quality offshore seawater to maintain mussels in flowing water for 24 h before and during SFG measurement. The seawater should be temperature controlled (preferably 15°C, a typical ambient temperature in early summer), aerated, and filtered (1 µm). When using recirculated seawater, any algal cells added to the water should be removed by filtration.

4) A constant temperature room for conducting the physiological measurements (e.g. 15°C).

5) A salinity meter and thermometer.

6) Algal culture providing high quality dividing algal cells to feed to mussels (e.g. unicellular algae such as *I. galbana*, *P. tricornutum*, or *T. suecica* with cells >4–6 µm spherical equivalent diameter to ensure 100% retention by the gills. Note that some cultures of *Isochrysis galbana* produce slightly smaller cells, and there is evidence that they may be retained with <100% efficiency by the gills of adult mussels.

7) Flow-through chambers (~500 ml) for holding and feeding individual mussels.

8) A peristaltic pump for dosing algal culture into the mixing chamber before delivery into individual mussel chambers (Figure 1A).

9) An electronic particle counter suitable for measuring algal cell concentrations (e.g. Coulter Counter).

10) 2 l tall form beakers (n = 17) for measuring the clearance rate in a closed system.

11) Multi-point solid state magnetic stirrers for 17 beakers and magnetic followers (2-cm length). Magnetic stirrers should be operated at a moderately high speed to ensure good mixing and a high current speed, which is preferred by mussels (Widdows *et al.*, 2002). However, the magnetic followers should not show irregular movement, detach from the magnet, or move the mussels.

12) A tray of 85 25-ml scintillation vials ((16 mussels + 1 control) × 5 samples).

13) Adjustable pipettes (200–1000 µl; 1–5 ml).

14) Oxygen electrodes (n = 8) and meters for measuring oxygen consumption (e.g. Strathkelvin Instruments) and eight respirometer chambers (e.g. Quickfit 500 ml volume) with an aperture in the lid to take the oxygen sensor, and a perforated base-plate to support the mussel above the magnetic follower (stirrer bar; Figure 1B).

15) A temperature-controlled water bath and multi-point magnetic stirrer (eight positions) for holding and mixing water in the respirometer chambers.

16) A drying oven at 100°C and furnace at 450°C to determine the dry and ash weight of food and faecal samples.
17) Electronic balance (five decimal places).

Generally two persons are required for field collection of mussels and measurement of SFG.

3 Methods

3.1 Collection of mussels

Intertidal mussels of standard shell length (4 cm) are collected from sites ranging from "clean" to "polluted". Avoid using large mature or senile mussels because their behaviour and pumping is slower and more erratic. One hundred fifty to two hundred individuals (i.e. 20 mussels for SFG and the remainder for chemical contaminant analysis) from each site are packed in an insulated container to keep them cool (~5°C) and damp while air-exposed during transportation to the laboratory (usually via commercial express delivery services in <24 h). The containers are cooled by ice packs, with absorbent material (such as disposable nappies) to protect the mussels from direct contact with the ice packs.

Intertidal mussels are preferable because they are adapted to a more dynamic environment and tend to tolerate gentle handling and prolonged air exposure better than subtidal mussels. As a result, they resume pumping activity more rapidly (within 15 min) following brief air exposure, thus providing a more consistent feeding rate. In contrast, subtidal mussels usually show more short-term variation in feeding rates owing to periods of reduced activity, making it more difficult to measure consistent feeding rates over a short period (i.e. 2–3 h). In addition, intertidal mussels can be transplanted to subtidal environmental locations for several weeks and retain the ability to close their valves during prolonged air exposure and tolerate disturbance and gentle handling.

3.2 Preparation of SFG mussels before physiological measurements

Open the insulated container and select 20 mussels of standard size (4 cm) and uniform shape (i.e. avoid any with deformed shells). Handle mussels gently to avoid excessive physical disturbance. Carefully clean shells of epibionts and sediment and rinse them briefly in a container of seawater. Avoid prolonged submersion in dirty water, as some may be gaping slightly after prolonged air exposure.

First, place the mussels in a closed tank (ca. 20 l volume) of aerated seawater at 15°C (i.e. in a temperature-controlled room or water bath) for ~1 h to allow them to flush out sediment and excretory products. At this stage, check for any spawning animals (isolate immediately and discard). Remove the mussels from tank and discard the water.

Before placing the mussels in flowing seawater to recover from the prolonged air exposure, dry them carefully with paper tissue, then place them on paper tissue to air dry for ca. 10 min. Number the individuals from 1 to 20 on both shell valves with a permanent marker pen (silver or gold Pilot or Pentel marker pens are ideal). Allow the marker ink to dry for 10 min and place in flowing seawater.

3.3 Preparation of the remaining mussels before chemical contaminant analysis

Clean the shells of the remaining mussels of sediment and place them in a 20 l closed tank of seawater for a period of 8 h to allow the discharge of sediment and faecal material from the mantle cavity and intestine. These are then either dissected directly into clean containers, or the mussels are frozen directly in their shells, and the samples are stored at −25°C (or ideally −80°C) for subsequent chemical analysis.

3.4 Standardized laboratory conditions

For the purposes of SFG measurement, mussels must be held under standardized laboratory conditions:

- clean and uncontaminated seawater, ideally collected offshore (30–34 salinity);
- filtered seawater (preferably to 1 μm);
- temperature controlled at 15°C.

Mussels should be fed a high quality culture of unicellular algae (e.g. *I. galbana* at a cell concentration of 15 000 cells ml^{-1} (or 0.43 mg algal cells l^{-1}). If other algal cells are used, cell concentrations should reflect their different cell sizes (e.g. dry weights of *I. galbana*, *P. tricornutum*, and *T. suecica* are ~30, 77, and 168 pg $cell^{-1}$ respectively; Brown, 1991).

These experimental conditions and the temperature of the respirometry water bath should be checked and recorded daily before each set of measurements.

3.5 Maintenance and feeding of mussels before SFG measurement

Before SFG measurement, mussels must be allowed to recover completely from the prolonged air exposure and transportation (e.g. 24 h). After the initial recovery phase (i.e. 1 h in a static tank), mussels are placed in individual flow-through chambers and allowed to recover and resume feeding during the afternoon and overnight. Animals 1 to 16 are placed in individual chambers, with flowing water of ca. 180–200 ml min^{-1} and algal concentration of 0.43 mg algal cells l^{-1} (15 000 cells ml^{-1} of *I. galbana*). Although sixteen individuals are routinely measured, at least four reserve mussels are held in a tank and fed from the outflow of the individual chambers in case any replacements are required (e.g. any failing to produce faeces or spawners).

At the end of the working day (i.e. after ca. 5 h of recovery in seawater), all faecal material should be siphoned out of the chambers and discarded. (The inorganic content of these initial faeces will be influenced by the previous environmental seston.) Waste faecal material should not be returned to the seawater system. If any mussels do not produce faeces at this stage, they should be replaced from the reserve group (i.e. animals 17–20). The mussels should then be maintained overnight with constant flowing water (180–200 ml min^{-1}) with algal food. An adjustable peristaltic pump is used to provide a constant supply of algal cells to the mussel chambers (Figure 1A) from a gently aerated 15 l vessel of algal culture. Adjust the algal pump dosing rate to achieve the required cell concentration (i.e. start low and increase gradually). Check cell concentration regularly to ensure a steady concentration has been achieved in the flow-through chambers. Use algae from the same high quality stock throughout the experiment. Clean the algal vessel daily and add new culture at the beginning of each morning to avoid using 24-h-old culture.

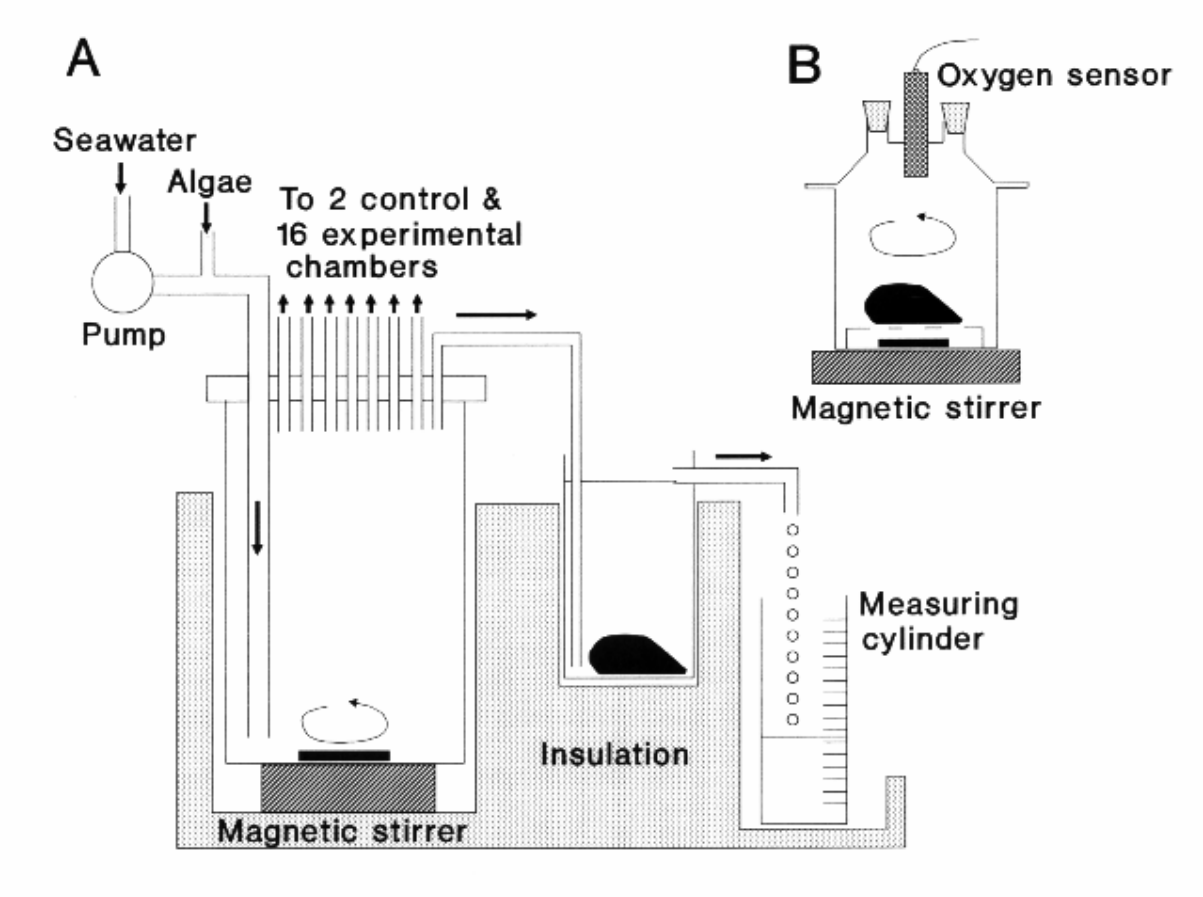

Figure 1A. Flow-through apparatus for maintaining and feeding of mussels.
Figure 1B. Glass respirometer for measurement of oxygen consumption.

If a flowing seawater system at 15°C is not available, or if exposure experiments are conducted when it is necessary to avoid using and disposing of large quantities of water and toxicant, then maintaining mussels in a smaller volume of seawater (20–50 l) in a constant temperature room is acceptable. However, it is necessary to change seawater at least daily to maintain adequate water quality. In these closed systems, it is difficult to maintain algal cell concentrations at a required level because it is not possible to match cell dosing (input) with the rate of filter feeding by the mussels (removal). Under these conditions, it is necessary to express food ration in terms of algal dry weight as a percentage of mussel dry body weight per day. The calculated total volume of algae stock required to provide a daily ration of ~8% of the total dry weight of all mussels in the tank is then continuously dosed into the closed tank over each 24-h period.

3.6 Collection of faecal material

On the following morning, the first task is to carefully collect the faeces (faecal pellets only). At this stage, mussels are carefully removed from the CR chambers and air exposed for 45 min on the bench (e.g. 9:00–9:45). This serves to 1) provide a period of air exposure for intertidal mussels, thus avoiding very constant conditions that are atypical of their natural environment and can induce more sluggish and inconsistent behaviour; and 2) permit faeces to be collected more easily.

3.7 Measurement of clearance rate

Clearance rate (CR), which is defined as the volume of water cleared of suspended particles (i.e. particles >4 µm equivalent spherical diameter) per hour, can be determined either in a

closed or a flow-through system (Annex 3) by measuring the removal of suspended algal cells added to filtered seawater (FSW down to 1 µm). Previous studies have shown that there are no significant differences between the methods (Widdows, 1985a). Although a flow-through system has been used routinely in field monitoring programmes (Widdows *et al.*, 1995a, 2002; Annex 3), most toxicological studies in the laboratory use a simple closed system to avoid consuming and disposing of large quantities of water containing toxic chemicals (see references in Annex 1 Table). The closed system of measurement, in which CR is calculated from the exponential decline in cell concentration in a beaker or tank of water over a period of 1.5 to 2 h, is recommended for simplicity.

Sixteen mussels are placed gently into separate beakers (a stainless steel spoon is useful for this), each containing 2 l of filtered seawater (FSW) and a magnetic stirrer bar (2 cm). An additional beaker without a mussel acts as a control. The water is mixed using a magnetic stirrer base plate. To avoid any physical disturbance, position each mussel to one side of the beaker away from the stirrer bar. An alternative and less costly method of mixing is to aerate each beaker. However, aeration is not recommended for toxicological exposure studies because more volatile toxicants are lost from seawater.

After a period of 15 min, to allow for the mussels to open their shell valves and resume pumping, algal culture is added to each beaker to give an initial concentration of 15 000 *Isochrysis* cells ml^{-1}, which then declines exponentially as a result of the filtration by the mussels. It is important not to exceed this maximum concentration to avoid pseudofaeces production and the inhibition of CR. Allow 5 min for the algal cells to be thoroughly mixed in the 2 l and then sample a 20-ml aliquot from the centre of each beaker in rapid succession (using a large syringe with an extension tube). Place these T_0 samples in numbered vials and count the cell concentrations (mean of 3 counts) using an electronic particle counter (e.g. Coulter Counter). Thereafter, take four 20-ml samples at 20-min intervals over a period of 1 h 20 min (e.g. 10:30 to 11:50). An alternative and less precise method is to measure the algal concentration using fluorescence spectrophotometry.

The CR by individual mussels is then calculated from the linear decline in log cell concentration over time, using the following equation (Coughlan, 1969):

$$CR\ (l\ h^{-1}) = (Vol) \times (\log_e C_1 - \log_e C_2) / \text{time interval in h}$$

where Vol is the volume of water (e.g. 2 l), and C_1 and C_2 are the cell concentrations at the beginning and end of each time increment (i.e. 20 min or 0.33 h). The control beaker should not show a significant change in cell concentration. However, if there are significant changes in the control (resulting mainly from settlement if the culture is in poor condition), then the control beaker must be replicated, the rate of decline calculated using the above equation, and then subtracted from the experimental rates.

The maximum clearance rate of each mussel is then calculated, based on a period of two consecutive time increments (i.e. 40 min), during which the decline in cell concentration was greatest. This avoids the inclusion of periods when individuals may be totally or partially closed (e.g. at the beginning, if slow to open, or at the end, if as a result of a high CR they have consumed most of the cells and thus switched off their ciliary pump. Refer to Annex 4, Examples of a Spreadsheet.

The following points should be noted:

- Large mussels (i.e. >4–5 cm) with high clearance rates will reduce the algal cell concentration in 2 l of seawater to <1000 cells ml^{-1} in less than 90 min. Such low concentrations are likely to inhibit CR. Consequently, when measuring high CR by larger individuals, it is advisable to use a larger volume of water (e.g. 5 l) rather than measure over a shorter period. However, larger 5 l containers will not fit readily on multi-point magnetic stirrers and aeration is the only practical

method of ensuring that the water is thoroughly mixed and the cell concentration is homogeneous.

- Ensure that the electronic particle counters are well maintained (i.e. clean and calibrated) and provide consistent counts and low counts for filtered seawater.
- It is important not to disturb mussels by either vibration or sudden shading by looking into chambers, while sampling from the CR beakers.
- Record all results on the data sheets and calculate CR (Annex 3).

3.8 Measurement of food absorption efficiency

Absorption efficiency is measured by the ratio method of Conover (1966); it represents the efficiency with which organic material is absorbed from the ingested food material.

$$\text{Absorption Efficiency} = (F - E) / [(1 - E)F]$$

where F = ash-free dry weight:dry weight ratio of food, and

E = ash-free dry weight:dry weight ratio of the faeces.

For the purposes of SFG standardization, including intra- and inter-laboratory comparison, it is necessary to use algal culture as food rather than natural seston because the latter will show spatial and temporal variation in quantity and quality. Although GFC filters are ideal for natural seston with high inorganic content, small disposable aluminium dishes are generally more reliable when measuring the small mass and the very low inorganic weights associated with uni-algal culture (food).

Whether collecting faeces and algal food on pre-ashed and weighed GFC filters or disposable aluminium dishes, both require careful preparation and handling and the use of blank weight corrections at all stages to avoid significant errors. Accurate weighing is essential; dry weights should be determined to ±0.02 mg using a calibrated balance.

Faeces are collected after mussels have been held in the laboratory at a constant algal cell concentration. However, the initial faecal deposits (i.e. production over the initial 5–6 h) are discarded to avoid contamination of faeces by previous diet (i.e. natural seston).

Faecal pellets are collected from the flow-through holding chambers with a wide mouth pipette (e.g. 10 ml adjustable with a disposable tip). Avoid breaking up faecal pellets by drawing them into the pipette slowly and carefully ejecting them into 50-ml centrifuge tubes. Pool faeces from groups of four or five mussels (to provide sufficient material), allow it to settle in tubes, and carefully draw off most of the seawater with a large-volume pipette.

Add 0.5 M ammonium formate (purest grade), allow the faeces to settle, and then draw off most of the fluid. Repeat twice to remove seawater salts from the faecal samples. Using centrifuge tubes with conical tapered bottoms allows withdrawal of seawater and subsequent washing with ammonium formate with minimum disturbance of the faecal pellets.

Algal food samples (ca. 1 l of culture) for absorption efficiency measurements can be taken from the algal dosing vessel, either at the beginning or the end of the faecal collection period. A small subsample can also be taken for particle counting (Coulter Model D) to relate dry weight to cell concentration. Spin down ca. 200 ml of algal culture (*I. galbana*) to produce an algal pellet (ca. 20 mg dry weight). Produce at least four replicate samples of algal food. Decant seawater and rinse gently with 0.5 M ammonium formate. Repeat centrifugation (ca. 6000 g for 15 min) and washing twice to remove seawater salts from algal samples.

Place the algal food samples and faecal samples from each group of mussels in separate pre-muffled and pre-weighed aluminium dishes. Dry at 100°C to constant weight (e.g. 2 d) and record the dry weights as soon as possible after cooling in a desiccator. Then place the samples in a cool furnace, bring to temperature, and ash at 450°C for 1 h. After cooling the

samples in a desiccator, weigh again and record the ashed weights. Cover the samples loosely with aluminium foil while they are in the oven and furnace to avoid dust contamination. Sample storage time in the desiccators before weighing should be minimized and silica gel replaced regularly.

An example of a spreadsheet for the calculation of food absorption efficiencies is presented in Annex 5.

3.9 Measurement of respiration rate

Rates of oxygen consumption by individual mussels are measured in closed glass respirometers (e.g. 500 ml Quickfit flasks) held in a temperature-controlled water bath mounted on a multi-point magnetic stirrer. Air-saturated seawater is added to each respirometer and stirred with a magnetic stirrer bar beneath a perforated glass plate (e.g. small Petri dish with many 1-cm holes) supporting a mussel (Figure 1B). The rate of decline in oxygen partial pressure (PO_2) in each chamber is measured by a calibrated oxygen electrode (e.g. Strathkelvin 1302) connected to an oxygen meter (e.g. Strathkelvin Model 781b). Eight respirometers are usually run simultaneously, and each oxygen meter is coupled to a multi-channel chart recorder or a PC. Twenty minutes are allowed for the mussels to open and to resume pumping, then oxygen uptake is measured for the next hour. The rate of oxygen consumption should not be measured below a partial pressure of ca. 100 mm Hg (13 kPa) because the rate then becomes dependent on the external PO_2.

The partial pressure of oxygen representing air saturation varies slightly as a function of temperature and atmospheric pressure according to the following equation (Gnaiger and Forstner, 1983):

$$PO_2 \text{ (mm Hg)} = [\text{Barometric press.} - (5.7 + 0.03 \times (\text{Temp } °C)^2)] \times 0.20946$$

e.g. 160 mm Hg = (776 mm Hg − (5.7 + 0.03 × 15^2)) × 0.20946.

[Conversion factors for pressure: 1 atm = 101.325 kPa; 1 mm Hg = 0.133322 kPa]

Oxygen solubility values are dependent on both temperature and salinity (Annex 6), and these are used to convert PO_2 (mm Hg) values to oxygen concentration in µmoles O_2 l^{-1} as follows:

$$C(t) = [(\text{Exptl. } PO_2 \text{ in mm Hg}) / (PO_2 \text{ at air saturation})] \times 259.6 \text{ µmoles } O_2\ l^{-1}$$

(e.g. 259.6 µmoles O_2 l^{-1} is concentration and 156.6 mm Hg is PO_2 at air saturation; when 15°C, 32 ppt and 760 mm Hg or 101.325 kPa; Annex 6).

The rate of oxygen consumption is then calculated as follows (Annex 7):

$$\text{Rate of } O_2 \text{ uptake (µmoles } O_2\ h^{-1}) = [C(t_0) - (C(t_1)] \times (V_r) \times 60/(t_1 - t_0)$$

where t_0, t_1 = start and finish times (min) of the measurement period;

$C(t)$ = concentration of oxygen in the water (µmoles O_2 l^{-1}) at time t;

V_r = volume of respirometer minus the animal.

The standard operating procedures and sequence of events are as follows:

Place a 5-l flask of seawater in a water bath and aerate (to achieve temperature equilibrium and full air saturation) for ca. 60 min (e.g. 9:00–10:00).

Fill the respirometer chambers and temperature equilibrate respirometers and oxygen sensors for >1 h before use (i.e. set them up in the morning, ready for use in the afternoon after CR measurements). Calibrate to full air saturation (e.g. ~158 mm Hg depending on barometric pressure).

After completion of the CR measurements (e.g. 14:00), gently remove eight mussels from the CR beakers for measurement of respiration rates (e.g. eight respirometry chambers with oxygen sensors are run simultaneously).

Place eight individual mussels in separate respirometers, partially emptying and refilling with the same water to ensure that there are no air bubbles within the chambers. Seal the chambers and measure the decline in oxygen concentration for at least 60 min. (Note the times and mm Hg at intervals. It is best to record the output on a PC or chart recorder.) Use only data from the linear decline in oxygen concentration. Do not include the initial period (~10 min) when there is a more rapid decline in oxygen caused by a disturbance of the sensor's temperature equilibration when adding the mussel and/or the release of deoxygenated water immediately after opening their valves.

Aerate another 5 l of seawater in the water bath ready for the next group of eight mussels.

After ca. 60–75 min, take final readings (time and mm Hg) and replace the seawater and mussels in respirometers. Repeat as above. Record the volumes of the respirometry chambers at end of the run by pouring water into the measuring cylinder.

Record all results on data sheets and calculate the respiration rate (Annex 7).

3.10 Use of oxygen sensors

Oxygen sensors are calibrated in solutions of known oxygen tension. After renewing the membrane on an oxygen sensor, it should be placed in PO_2 zero solution, and the meter adjusted to zero. Before using the probe, it should be left to stabilize for >12 h with the polarizing current on. Each day, the probe should be calibrated in air-saturated (i.e. aerated) seawater at the required experimental temperature. The oxygen meter is then set at the appropriate PO_2 for air saturation (see above) when they have stabilized (i.e. become temperature and oxygen equilibrated).

A silicone tubing sleeve (or several layers of parafilm wrapped around the sensor) provides a seal between the sensor and the orifice in the respirometer chamber. However, this should be kept to a minimum to avoid diffusion of oxygen into the respirometer.

3.11 Measurement of mussel dry weights

After completing the physiological measurements, record the shell length of each mussel, remove tissues from the shells (draining seawater from the mantle cavity), and place the tissue in a pre-weighed foil cup. Dry to constant weight at 100°C (ca. 2 d). Record dry tissue weight.

4 Calculation of scope for growth

After all physiological measurements have been completed, the physiological rates are corrected to a "standard body size" (e.g. 1 g dry weight) using appropriate weight exponents (e.g. $b = 0.67$; Annex 8). The measured physiological responses are then converted into energy equivalents (J g^{-1} h^{-1}) and used in the balanced energy equation to calculate the energy available for growth and reproduction (i.e. SFG).

C = Energy consumed or ingested

C = [maximum clearance rate: l g^{-1} h^{-1}] × [mg POM l^{-1}] × [23 J mg^{-1} POM]

where the energy content of particulate organic matter (POM) or algal food is 23 J mg^{-1} ash-free POM (Slobodkin and Richman, 1961; Widdows *et al.*, 1979). For example, a standardized algal cell concentration of 0.435 mg POM l^{-1} × (23 J mg^{-1} POM) = 10 J l^{-1}.

A = Energy absorbed

A = (C) × food absorption efficiency.

R = Energy respired

R = (µmoles O_2 g^{-1} h^{-1}) × 0.456

where the heat equivalent of oxygen uptake is 0.456 J µmole^{-1} O_2 (Gnaiger, 1983).

P = Scope for Growth

P = A – R.

SFG values for a particular site or experimental treatment are then expressed as mean ± 95% CI (n = 16; Annex 9).

5 References

Anderlini, V. C. 1992. The effect of sewage on trace metal concentrations and scope for growth in *Mytilus edulis aoteanus* and *Perna canaliculus* from Wellington Harbour, New Zealand. Science of the Total Environment, 125: 263–288.

Bayne, B. L., Clarke, K. R., and Moore, M. N. 1981. Some practical considerations in the measurement of pollution effects on bivalve molluscs, and some possible ecological consequences. Aquatic Toxicology, 1: 159–174.

Bayne, B. L., and Widdows, J. 1978. The physiological ecology of two populations of *Mytilus edulis* L. Oecologia, 37: 137–162.

Brown, M. R. 1991. The amino acid and sugar composition of 16 species of microalgae used in aquaculture. Journal of Experimental Marine Biology and Ecology, 145: 79–99.

Butler, R., Roddie, B. D., and Mainstone, C. P. 1990. The effects of sewage sludge on two life history stages of *Mytilus edulis*. Chemistry and Ecology, 4: 211–219.

Conover, R. J. 1966. Assimilation of organic matter by zooplankton. Limnology and Oceanography, 11: 338–354.

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

Crowe, T. P., Smith, E. L., Donkin, P., Barnaby, D. L., and Rowland, S. J. 2004. Measurement of sublethal stress effects on individual organisms indicate community-level impacts of pollution. Journal of Applied Ecology, 41: 114–123.

Donkin, P., and Widdows, J. 1990. Quantitative structure–activity relationships in aquatic invertebrate toxicology. Review in Aquatic Sciences, 2: 375–398.

Donkin, P., Widdows, J., Evans, S. V., Worrall, C. M., and Carr, M. 1989. Quantitative structure-activity relationships for the effect of hydrophobic organic chemicals on rate of feeding by mussels *Mytilus edulis*. Aquatic Toxicology, 14: 277–294.

Donkin, P., Widdows, J., Evans, S. V., and Brinsley, M. D. 1991. QSARs for the sublethal responses of marine mussels *Mytilus edulis*. Science of the Total Environment, 109/110: 461–476.

Donkin, P., Widdows, J., Lowe, D. M., Donkin, M. E., and Price, D. N. 1996. Sublethal effects of waste on marine organisms: responses measured at the whole organism level. *In* Toxic Impacts of Wastes on the Aquatic Environment, pp. 138–145. Ed. by J. F. Tapp, J. R. Wharfe, and S. M. Hunt. The Royal Society of Chemistry, London.

Donkin, P., Widdows, J., Evans, S. V., Staff, F. J., and Yan, T. 1997. Effect of neurotoxic pesticides on the feeding rate of marine mussels *Mytilus edulis*. Pesticide Science, 49: 196–209.

Donkin, P., Smith, E. L., and Rowland, S. J. 2003. Toxic effects of unresolved complex mixtures of aromatic hydrocarbons accumulated by mussels, *Mytilus edulis*, from contaminated sites. Environmental Science and Technology, 37: 4825–4830.

Gilfillan, E. S., Mayo, D. W., Page, D. S., Donovan, D., and Hanson, S. 1977. Effects of varying concentrations of petroleum hydrocarbons in sediment on carbon flux in *Mya arenaria*. *In* Pollution and Physiology of Marine Organisms, pp. 421–438. Ed. by W. B. Vernberg, A. Calabrese, F. P. Thurberg, and F. J. Vernberg. Belle Baruch Library in Marine Science, University of South Carolina Press, Columbia.

Gnaiger, E. 1983. Heat dissipation and energetic efficiency in animal anoxibiosis: economy contra power. Journal of Experimental Zoology, 228: 471–490.

Gnaiger, E., and Forstner, H. 1983. Polarographic Oxygen Sensors: Aquatic and Physiological Applications. Springer-Verlag, Berlin.

Granmo, Å., Ekelund, R., Magnusson, K., and Bergren, M. 1989. Lethal and sublethal toxicity of 4-Nonylphenol to the common mussel *Mytilus edulis* L. Environmental Pollution, 59: 115–127.

Halldórsson, H. P., Svavarsson, J., and Granmo, Å. 2005. The effect of pollution on scope for growth of the mussel (*Mytilus edulis* L.) in Iceland. Marine Environmental Research, 59: 47–64.

Hildreth, D. I., and Crisp, D. J. 1976. A corrected formula for calculation of filtration rate of bivalve molluscs in an experimental flowing system. Journal of the Marine Biological Association UK, 56: 111–120.

Kautsky, N., Johanneson, K., and Tedengren, M. 1990. Genotypic and phenotypic differences between Baltic and North Sea populations of *Mytilus edulis* evaluated through reciprocal transplantations. 1. Growth and Morphology, Marine Ecology Progress Series, 59: 203–210.

Kiørboe, T., Møhlenberg, F., and Nohr, O. 1980. Feeding, particle selection and carbon absorption in *Mytilus edulis* in different mixtures of algae and resuspended bottom material. Ophelia, 19: 193–205.

Martin, M., and Severeid, R. 1984. Mussel watch monitoring for the assessment of trace toxic contaminants in California marine waters. *In* Concepts in Marine Pollution Measurements, pp. 291–323. Ed. by H. H. White. Maryland Sea Grant College, University of Maryland.

Møhlenberg, F., and Riisgard, H. U. 1978. Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia, 17: 239–246.

Navarro, J. M., and Widdows, J. 1997. Feeding physiology of *Cerastoderma edule* in response to a wide range of seston concentrations. Marine Ecology Progress Series, 152: 175–186.

Nelson, W. G. 1990. Use of the blue mussel, *Mytilus edulis*, in water quality toxicity testing and *in situ* marine biological monitoring. *In* Aquatic Toxicology and Risk Assessment, pp.167–175. Ed. by W. G. Landis and W. H. Schalie. Vol. 13, ASTM STP 1096. American Society for Testing and Materials, Philadelphia.

Page, H. M., and Hubbard, D. M. 1987. Temporal and spatial patterns of growth in mussels *Mytilus edulis* on an offshore platform–relationships to water temperature and food availability. Journal of Experimental Marine Biology and Ecology, 111: 159–179.

Poulson, E., Riisgard, H. U., and Møhlenberg, F. 1982. Accumulation of cadmium and bioenergetics in the mussel, *Mytilus edulis*. Marine Biology, 68: 25–29.

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

Slobodkin, L. B., and Richman, S. 1961. Calories/gram in species of animals. Nature, 191: 299.

Widdows, J. 1976. Physiological adaptation of *Mytilus edulis* to cyclic temperatures. Journal of Comparative Physiology, 105: 115–128.

Widdows, J. 1978. Physiological indices of stress in *Mytilus edulis*. Journal of the Marine Biological Association UK, 58: 125–142.

Widdows, J. 1978a. Combined effects of body size, food concentration, and season on the physiology of *Mytilus edulis*. Journal of the Marine Biology Association UK, 58: 109–124.

Widdows, J. 1978b. Physiological indices of stress in *Mytilus edulis*. Journal of the Marine Biology Association UK, 58: 125–142.

Widdows, J. 1983. Field measurement of the biological effects of pollutants. *In* Proceedings of a Pacific Regional Workshop on Assimilative Capacity of the Ocean's for Man's Wastes,

pp. 111–129. Ed. by Jong-Chin Su and Tzu-Chang Hung. SCOPE/ICSU Academia Sinica, Taipei.

Widdows, J. 1985a. Physiological procedures. *In* The Effects of Stress and Pollution on Marine Animals, pp. 161–178. Praeger Press, New York.

Widdows, J. 1985b. The effects of fluctuating and abrupt changes in salinity on the performance of *Mytilus edulis* L. *In* Marine Biology of Polar Organisms and Effects of Stress on Marine Organisms, pp. 555–566. Ed. by J. S. Gray and M. E. Christiansen. J. Wiley, Chichester.

Widdows, J. 1993. Marine and estuarine invertebrate toxicity tests. *In* Handbook of Ecotoxicology, Volume 1, Chapter 9, pp. 145–166. Ed. P. Calow. Blackwell, Oxford.

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

Widdows, J., and Donkin, P. 1991. Role of physiological energetics in ecotoxicology. Comparative Biochemistry and Physiology, 100C: 69–75.

Widdows, J., and Donkin, P. 1992. Mussels and environmental contaminants: bioaccumulation and physiological aspects. *In* The mussel *Mytilus*, Chapter 8, pp. 383–424. Ed. by E. Gosling. Elsevier Press, Amsterdam.

Widdows, J., and Johnson, D. 1988. Physiological energetics of *Mytilus edulis*: Scope for growth. Marine Ecology Progress Series, 46: 113–121.

Widdows, J., and Page, D. S. 1993. Effects of tributyltin and dibutyltin on the physiological energetics of the mussel, *Mytilus edulis*. Marine Environmental Research, 35: 233–249.

Widdows, J., and Staff, F. J. 1997. Practical procedures for the measurement of scope for growth. Plymouth Marine Laboratory, 48 pp.

Widdows, J., Moore, M. N., Lowe, D. M., and Salkeld, P. N. 1979. Some effects of a dinoflagellate bloom *Gyrodinium aureolum* on the mussel *Mytilus edulis*. Journal of the Marine Biological Association UK, 59: 522–524.

Widdows, J., Phelps, D. K., and Galloway, W. 1981. Measurement of physiological condition of mussels transplanted along a pollution gradient in Narragansett Bay. Marine Environmental Research, 4: 181–194.

Widdows, J., Bakke, T., Bayne, B. L., Donkin, P., Livingstone, D. R., Lowe, D. M., Moore, M. N., Evans, S. V., and Moore, S. L. 1982. Responses of *Mytilus edulis* on exposure to the water-accommodated fraction of North Sea oil. Marine Biology, 67: 15–31.

Widdows, J., Donkin, P., Salkeld, P. N., and Evans, S. V. 1987. Measurement of scope for growth and tissue concentrations of mussels *Mytilus edulis* at sites in the vicinity of Sullom Voe: a case study. *In* Fate and Effects of Oil in the Marine Ecosystems, pp. 269–277. Ed. by J. Kuiper and W. J. van den Brink. Nijhoff, Dordrecht.

Widdows, J., Burns, K. A., Menon, N. R., Page, D., and Soria, S. 1990. Measurement of physiological energetics, scope for growth, and chemical contaminants in mussel *Arca zebra* transplanted along a contamination gradient in Bermuda. Journal of Experimental Marine Biology and Ecology, 138: 99–117.

Widdows, J., Donkin, P., Brinsley, M. D., Evans, S. V., Salkeld, P. N., Franklin, A., Law, R. J., and Waldock, M. J. 1995a. Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. Marine Ecology Progress Series, 127: 131–148.

Widdows, J., Donkin, P., Evans, S. V., Page, D. S., and Salkeld, P. N. 1995b. Sublethal biological effects and chemical contaminant monitoring of Sullom Voe (Shetland), using mussels (*Mytilus edulis*). Proceedings of the Royal Society of Edinburgh, 103B: 99–112.

Widdows, J., Nasci, C., and Fossato, V. U. 1997. Effects of pollution on the scope for growth of mussels *Mytilus galloprovincialis* from the Venice Lagoon. Marine Environmental Research, 43: 69–79.

Widdows, J., Donkin, P., Staff, F. J., Matthiessen, P., Law, R. J., Allen Y. T., Thain, J. E., Allchin, C. R., and Jones, B. R. 2002. Measurement of stress effects scope for growth. and contaminant levels in mussels *Mytilus edulis* collected from the Irish Sea. Marine Environmental Research, 53: 327–356.

Annex 1: Examples of the application of scope for growth

Scope for growth has been applied in laboratory and mesocosm experiments to assess the toxic effects (from sublethal to lethal) of a range of environmentally important chemical contaminants, including aromatic and aliphatic hydrocarbons (Widdows *et al.*, 1982; Donkin *et al.*, 1989, 1991), sewage sludge (Butler *et al.*, 1990), tri- and dibutyltin (Widdows and Page, 1993), nonylphenol (Granmo *et al.*, 1989), pentachlorophenol (Widdows and Donkin, 1991), and organochlorine, organophosphate, and pyrethroid pesticides (Donkin *et al.*, 1997). These laboratory studies have been particularly important in 1) establishing concentration–response relationships between the concentration of contaminants in the body tissues and the physiological responses of mussels, including SFG; 2) utilizing a quantitative structure–activity relationship (QSAR) approach to study the sublethal toxicity of organic contaminants (Donkin and Widdows, 1990). Such laboratory-derived concentration–response relationships have been used subsequently to provide a quantitative toxicological interpretation of field-derived SFG measurements and tissue residue chemistry.

There are many examples of the field application of SFG measurements combined with tissue residue chemistry as a means of assessing environmental pollution. These include studies of pollution gradients in Maine (Gilfillan *et al.*, 1977), Narragansett Bay (Widdows *et al.*, 1981), San Francisco Bay (Martin and Severeid, 1984), at the North Sea oil terminal in Sullom Voe, Shetlands (Widdows *et al.*, 1987, 1995b), Venice Lagoon (Widdows *et al.*, 1997), and two IOC (Intergovernmental Oceanographic Commission) GEEP Workshops concerned with contaminant gradients in a Norwegian fjord (Widdows and Johnson, 1988) and Bermuda (Widdows *et al.*, 1990). In addition, two field studies by Nelson (1990) and Anderlini (1992) have used SFG to assess the impact of sewage inputs to Narragansett Bay (USA) and Wellington Harbour (New Zealand), respectively.

More recently, the combined measurement of SFG and chemical contaminants in mussels has been successfully extended and applied over a larger spatial scale of >1000 km of North Sea coastline (Widdows *et al.*, 1995a) and Irish Sea coastline (Widdows *et al.*, 2002). The main features of the approach in the North Sea and Irish Sea studies were to: 1) identify regions as well as specific sites that were significantly stressed by pollutants; 2) quantify the degree of sublethal stress and how near the animals were to the lethal limit; 3) provide a quantitative toxicological interpretation of much of the contaminant data.

Therefore, these various field studies have demonstrated that this approach is able to detect and quantify changes in environmental quality, as well as identify some of the cause(s) of these changes through use of QSAR relationships and established cause–effect relationships (i.e. between contaminant concentrations in mussel tissues and the SFG response).

SFG has also been shown to be a sensitive and ecologically meaningful biological response that can provide a powerful, rapid (i.e. results can be obtained within days of sampling), and cost-effective method for monitoring changes in environmental quality (Widdows *et al.*, 1995a, 2002; Crowe *et al.*, 2004).

TOXICANT	REFERENCE	TISSUE CONCENTRATION INDUCING 50% REDUCTION IN FEEDING RATE (CR) OR SFG (µg g^{-1} dry weight)
HMW Alkanes	Widdows and Donkin (1992)	>2000 (CR)
HMW Aromatics	Widdows and Donkin (1992)	>2000 (CR)
Lindane	Donkin *et al.* (1997)	1400 (CR)
Cd	Poulson *et al.* (1982)	>150 (SFG)
Di(ethylhexyl)phthalate	Donkin *et al.* (1996)	330 (CR)
LMW Alkanes	Donkin *et al.* (1989)	125 (CR)
LMW Aromatics	Donkin *et al.* (1989) Widdows *et al.* (1995a)	125 (CR) 20 (SFG)
Carbaryl	Donkin *et al.* (1997)	50 (CR)
Pentachlorophenol	Widdows and Donkin (1991)	45 (SFG)
Cu	Widdows and Johnson (1988)	30 (SFG)
Tributyltin	Widdows and Page (1993)	4 (SFG)
Dichlorvos	Donkin *et al.* (1996)	2.2 (CR)

Annex 2: The justification for measuring scope for growth rather than simply clearance rate

The suspension feeding rate (i.e. CR) of bivalves is an important component in the calculation of the energy budget and hence SFG. It is also very responsive to a wide range of chemical contaminants (including metals, organo-metals, hydrocarbons, and pesticides) and, therefore, will reflect changes in water quality and the degree of pollution. However, the determination of CR as a single physiological parameter does not have the same power of discrimination as the more complete and integrated measure, SFG. The reasons for this are outlined below:

1) SFG is relatively independent of seasonal cycles in growth/gametogenesis and temperature (particularly during the summer growth period). The physiological responses such as CR and respiration rate follow a slight but significant seasonal cycle, which is primarily related to the gametogenic/nutritional storage cycle. There is a seasonal maximum weight-specific rate for both CR and respiration rate in spring, when mussels contain mature gametes, just before spawning. The mass specific rates gradually decline during summer as reserves are built up and metabolic rates reach minimum values in autumn, when they enter a period of quiescence before the onset of gametogenesis. As a direct result of the parallel change in both of these components, SFG (which is basically the difference between the two rates representing energy acquisition and energy utilization) is maintained independent of the seasonal cycles in growth/gametogenic and temperature (Widdows, 1978; Bayne and Widdows, 1978; Page and Hubbard, 1987). Consequently, CR can be used to quantify changes in water quality over relatively small spatial scales (i.e. within an estuary) and short time scales (i.e. within 2 or 3 w). However, when measuring over larger spatial scales (i.e. >50 km of coastline) and temporal scales (i.e. over weeks, months, and year-to-year), population differences in the timing of spawning/nutritional storage cycles can create significant additional variability in the CR. It is generally recommended that the measurement of SFG of mussels is confined to the summer period (June through September) because the spring-spawning and the autumn period of quiescence increase the variability/noise in all physiological responses (CR, respiration rate, and SFG). Therefore, SFG gets closer to an absolute measure of water quality that allows comparisons within and between studies.

2) The intra-population variability in the individual SFG data tends to be slightly lower than for CR, thus improving the ability to detect significant differences between populations/sites. This is the result of the degree of coupling between CR and respiration rate, for example, an individual with a lower CR will also tend to have a lower respiration rate (owing to lower metabolic costs associated with the ciliary pump (small), digestion, and protein synthesis (large)). However, there are occasions when changes in CR and respiration rates are not closely correlated, and this probably indicates a toxic effect on respiration (e.g. uncoupling of oxidative phosphorylation).

3) SFG provides an expansion of scale. CR generally ranges from ca. 6 l g^{-1} h^{-1} for mussels living in relatively clean environments, down to ca. 3 l g^{-1} h^{-1} in the more polluted coastal environments (although greater inhibitory effects on CR have been recorded in heavily polluted estuarine situations). When CR is converted to energy acquisition and the energy expenditure (i.e. respiration) is subtracted, this range from 6–3 l g^{-1} h^{-1} corresponds to a range of SFG from >20 J g^{-1} h^{-1} to <2 J g^{-1} h^{-1}. This expansion of scale reflects SFG's practice of not starting from zero but from ca. −10 J g^{-1} h^{-1} (i.e. the energy expenditure/costs under starvation conditions, before considering any input from the food). The increased scaling and discrimination provided by SFG is demonstrated by the mussels transplanted to offshore North Sea Lightvessels (Widdows *et al.*, 1995a). There was a statistically significant decline in SFG along the Humber plume (mean ±95% CI from 14.91 ± 2.28 to 10.29 ± 1.65 J g^{-1} h^{-1}), whereas the decline in CR was less marked and not statistically significant (mean ±95% CI from 3.36 ± 0.4 to 3.08 ± 0.34 l g^{-1} h^{-1}).

4) SFG response is inherently more meaningful than CR. The presentation of a pollution response in terms of a reduction in growth potential and the proximity to the zero SFG (i.e. the incipient lethal limit or the level at which the animal starts utilizing body reserves in order to survive) can be readily understood by the general public as well as environmental managers and scientists. In contrast, the CR response presented in terms of $l\ h^{-1}$ is not so readily interpreted.

5) Cost–benefit analysis of SFG measurements. Compared with CR, the principal disadvantage of SFG is the extra time/effort/equipment required. However, this is relatively small considering the investment of time and effort in collecting and transporting the samples (1 d), recovering mussels (1 d), and measuring their CR (3–4 h in the morning). The subsequent measurement of respiration rate (and absorption efficiency if not adopting a standard value) is a relatively small extra investment (i.e. 3 h in the afternoon), considering the additional benefits obtained (e.g. information on mechanisms of toxicity, reduction in seasonal variability, and improved detection, discrimination, and interpretation).

Annex 3: Flow-through system for clearance rate measurements

There are several publications that have made inaccurate and misleading statements about the measurement of CR in flow-though conditions (Riisgard, 2001; reply Widdows, 2001).

It has long been recognized that certain conditions should be met (Widdows, 1985a), and these have not always been clearly understood and acknowledged in later publications, some of which have been unduly critical of the method.

Accurate estimates of CR in flow-through systems are only achieved by:

1) Using appropriate flow rates, low enough to record a significant difference between the inflow and outflow cell concentration, yet sufficient to prevent any significant recirculation of water by the mussel in a small chamber. As a general guide, flow rates through each chamber should be approximately 2–3 times the clearance rate of the mussels (i.e. the cell concentration in the outflow should be >50% and <80% of the inflow concentration).

2) Using small-volume cylindrical chambers (i.e. of a size sufficient to hold the mussels), which provide short residence times.

3) Ensuring that the inflow water enters at the base of the chamber and next to the inhalant mantle edge of the mussel and that the outflow is at the top.

These conditions (Figure 1A) avoid any significant recirculation of the water by the mussel, and the CR is calculated by dividing the difference between inflow (C_I) and outflow (C_O) by the inflow cell concentration (C_I).

(1) Clearance rate ($l\ h^{-1}$) = Flow rate (in $l\ h^{-1}$) × $(C_I - C_O) / C_I$

An alternative method, proposed by Hildreth and Crisp (1976), calculates CR by dividing the difference between inflow and outflow by the outflow cell concentration.

(2) Clearance rate ($l\ h^{-1}$) = Flow rate (in $l\ h^{-1}$) × $(C_I - C_O) / C_O$

When using this equation, however, it is essential that the chamber containing the mussel should have a larger volume (~1 l) and the water should be thoroughly mixed by a stirrer or aeration to ensure the cell concentration in the outflow water is equivalent to that surrounding the mussel. Otherwise, the CR will be overestimated.

To ensure that all the appropriate conditions are satisfied for measurement of CR in a flow-through system, it is important to check against CR measurements in a closed system.

Standard Operating Procedures

After placing the mussels in the experimental chambers, (a stainless steel spoon is useful for this), they are left undisturbed for ~60 min to allow their valves to open and feeding to be resumed.

The flow-through CR measuring system (Figure 1A) consists of a small centrifugal pump (or a reservoir of sufficient height/pressure) discharging FSW into a mixing chamber (1.5 l volume) with a magnetic stirrer and thence via narrow (i.e. 2.5 mm) bore tubing through 18 identical chambers in parallel (16 experimental chambers with individual mussels and two control chambers without mussels).

Flow rates through each chamber are maintained constant at ca. 180–200 ml min^{-1}. The inflow into each chamber is at the bottom, next to the mussel's inhalant mantle edge, and the outflow is via an overflow tube at the top of the chamber (i.e. 400 ml of water in a 500-ml chamber). A variable speed peristaltic pump introduces algal cells into the mixing chamber to achieve the

required cell concentration (e.g. 15 000 *Isochrysis* cells ml^{-1}). Check the cell concentration regularly to ensure a steady-state has been achieved. Check the peristaltic pump tubing daily for wear, and replace or move along to a fresh section at regular intervals. (This is the most likely source of variability in the algal cell concentration in the chambers.)

Water samples from all chambers are collected simultaneously by moving a rack of measuring cylinders (>200 ml volume) directly under the outflows and removing them after a period of 60 s. It is important to avoid disturbing the animals by shading or vibration near the apparatus. The flow rate through each chamber is recorded. The concentration of algal cells in each water sample is then measured, using an electronic particle counter (e.g. Coulter Counter) with a 100- or 140-µm orifice tube and set to count all particles >3 µm in diameter (spherical equivalent) in a 0.5-ml subsample. Three or four replicate counts are made on each sample, and the mean calculated. Water samples for CR measurements are collected every 45 min over a period of 3 h. The maximum clearance rate of each mussel over two consecutive periods (i.e. 90 min) is then used in the calculation of SFG. This avoids the inclusion of periods when individuals may be totally or partially closed.

Annex 4: Example of a spreadsheet for calculating clearance rate in a closed system

Note that sampling time 5 was not used.

Sample: 1
Date: 18th. May, 1989.

ALGAL CELL NUMBERS / 0.5 ML DETERMINED USING COULTER COUNTER :-

TIME 0	COUNTS			MEAN	TIME 1	COUNTS			MEAN	TIME 2	COUNTS			MEAN
ANIMAL	1	2	3		ANIMAL	1	2	3		ANIMAL	1	2	3	
1	11832	11531	11597	11653.3	1	7410	7208	7240	7286.0	1	4417	4422	4604	4481.0
2	12166	12239	12239	12214.7	2	6360	6394	6343	6365.7	2	3323	3324	3399	3348.7
3	11104	11348	11156	11202.7	3	6337	6516	6433	6428.7	3	4005	4090	4119	4071.3
4	10245	10502	10617	10454.7	4	5655	5497	5604	5585.3	4	3671	3842	3840	3784.3
5	11169	11175	11078	11140.7	5	6514	6392	6507	6471.0	5	3103	3021	3038	3054.0
6	12923	12991	12655	12856.3	6	8509	8398	8510	8472.3	6	4835	4707	4973	4838.3
7	12488	12554	12452	12498.0	7	6414	6118	6282	6271.3	7	2709	2616	2919	2748.0
8	10772	10657	10752	10727.0	8	6842	6674	6540	6685.3	8	4008	3968	3954	3976.7
9	11791	11761	11913	11821.7	9	6482	6198	6426	6368.7	9	2899	2817	2830	2848.7
10	12069	12015	12053	12045.7	10	7469	7469	7465	7467.7	10	3842	3904	3887	3877.7
11	11988	11912	11986	11962.0	11	6875	6807	6670	6784.0	11	3817	3802	3674	3764.3
12	11656	11733	11739	11709.3	12	6463	6431	6571	6488.3	12	3321	3091	3014	3142.0
13	11511	11382	11606	11499.7	13	6357	6555	6354	6422.0	13	2944	3046	2931	2973.7
14	12428	12582	12512	12507.3	14	7721	7606	7501	7609.3	14	5091	5030	5039	5053.3
15	11341	11456	11265	11354.0	15	5758	5655	5597	5670.0	15	2577	2500	2575	2550.7
16	12950	12905	12919	12924.7	16	7437	7363	7366	7388.7	16	4569	4662	4658	4629.7
CONT	12725	12729	12736	12730.0	CONT	12760	12933	12688	12793.7	CONT	12726	12971	12758	12818.3

TIME 3	COUNTS			MEAN	TIME 4	COUNTS			MEAN	TIME 5	COUNTS			MEAN
ANIMAL	1	2	3		ANIMAL	1	2	3		ANIMAL	1	2	3	
1	2753	2688	2749	2730.0	1	1483	1439	1366	1429.3	1				
2	1906	1940	1933	1926.3	2	965	907	865	912.3	2				
3	2160	2153	2151	2154.7	3	1124	1057	1101	1094.0	3				
4	1314	1183	1227	1241.3	4	552	537	538	542.3	4				
5	1620	1481	1473	1524.7	5	788	792	739	773.0	5				
6	2623	2490	2511	2541.3	6	1497	1380	1473	1450.0	6				
7	1158	1160	1114	1144.0	7	469	487	510	488.7	7				
8	2566	2515	2417	2499.3	8	2058	1984	1968	2003.3	8				
9	1460	1407	1399	1422.0	9	796	763	780	779.7	9				
10	1991	1984	1981	1985.3	10	1048	1046	1043	1045.7	10				
11	1913	1914	1876	1901.0	11	1031	1050	1013	1031.3	11				
12	1541	1481	1515	1512.3	12	768	773	748	763.0	12				
13	1460	1553	1489	1500.7	13	731	737	676	714.7	13				
14	2802	2757	2684	2747.7	14	1672	1518	1492	1560.7	14				
15	1160	1153	1110	1141.0	15	623	617	589	609.7	15				
16	2501	2480	2452	2477.7	16	1234	1299	1221	1251.3	16				
CONT	12581	12568	12559	12569.3	CONT	12767	12456	12525	12582.7	CONT				

CLEARANCE RATE CALCULATION :-
Time interval = **20** minutes. **Vol. =** **2** litres.

CLEARANCE RATE CALCULATION :-
Time interval = **20** minutes. **Vol. =** **2** litres.

CR (L/h) / 1 INTERVAL						CR (L/h) / 2 INTERVALS				MAX CR (L/h) 2 INTERVALS	
ANIMAL	T0-T1	T1-T2	T2-T3	T3-T4	T4-T5	T0-T2	T1-T3	T2-T4	T3-T5		
1	2.82	2.92	2.97	3.88	-15.83	2.87	2.94	3.43		1	3.43
2	3.91	3.85	3.32	4.48	-18.52	3.88	3.59	3.90		2	3.90
3	3.33	2.74	3.82	4.07	-17.44	3.04	3.28	3.94		3	3.94
4	3.76	2.34	6.69	4.97	-21.65	3.05	4.51	5.83		4	5.83
5	3.26	4.51	4.17	4.08	-19.52	3.88	4.34	4.12		5	4.34
6	2.50	3.36	3.86	3.37	-15.75	2.93	3.61	3.62		6	3.62
7	4.14	4.95	5.26	5.10	-22.27	4.54	5.10	5.18		7	5.18
8	2.84	3.12	2.79	1.33	-13.81	2.98	2.95	2.06		8	2.98
9	3.71	4.83	4.17	3.61	-19.47	4.27	4.50	3.89		9	4.50
10	2.87	3.93	4.02	3.85	-17.71	3.40	3.97	3.93		10	3.97
11	3.40	3.53	4.10	3.67	-17.79	3.47	3.82	3.88		11	3.88
12	3.54	4.35	4.39	4.10	-19.60	3.95	4.37	4.25		12	4.37
13	3.50	4.62	4.10	4.45	-19.99	4.06	4.36	4.28		13	4.36
14	2.98	2.46	3.66	3.39	-15.30	2.72	3.06	3.52		14	3.52
15	4.17	4.79	4.83	3.76	-20.94	4.48	4.81	4.29		15	4.81
16	3.36	2.80	3.75	4.10	-16.63	3.08	3.28	3.92		16	3.92
CONT	-0.03	-0.01	0.12	-0.01	-2.78	-0.02	0.05	0.06			

SUMMARY OF RESULTS :-

N	=	**16**
MEAN	=	**4.16**
S.E.	=	**0.18**
2 S.E.	=	**0.35**
LOWER 95%C.I.	=	**3.81**
UPPER 95%C.I.	=	**4.51**

Annex 5: Example of spreadsheet for calculating food absorption efficiency

Calculation of Food Absorption Efficiencies

Experiment: ***Wash Study.***
Sample: ***Site H.***
Date: ***August 1995.***

Blank

Crucible or Aluminium foil No.	Initial wt. of foil (mg)	Dry wt. of sample + foil (mg)	Dry wt of sample only (mg)	Ash wt. of sample + foil (mg)	Ash wt. of sample only (mg)
1	12095.00	12095.28	0.28	12095.26	0.26
2	12016.95	12017.23	0.28	12017.25	0.30
3	10584.89	10585.40	0.51	10585.31	0.42
4	11595.32	11595.86	0.54	11595.77	0.45
5	12020.43	12020.81	0.38	12020.85	0.42
mean	11662.52		0.40	11662.89	0.37

Food

Crucible or Foil No.	Initial wt. of foil (mg)	Dry wt. of sample + foil (mg)	Dry wt of sample only (mg)	Dry wt. after blank correction (mg)	Ash wt. sample + foil (mg)	Ash wt of sample only (mg)	Ash wt. after blank correction (mg)	Food Value
6	11110.63	11130.87	20.24	19.84	11112.38	1.75	1.38	0.93
7	11600.31	11625.52	25.21	24.81	11602.12	1.81	1.44	0.94
8	12019.88	12031.17	11.29	10.89	12021.32	1.44	1.07	0.90
9	10135.27	10147.37	12.10	11.70	10136.53	1.26	0.89	0.92
10	12222.66	12249.34	26.68	26.28	12225.38	2.72	2.35	0.91
mean			19.10	18.71		1.80	1.43	**0.92**

Faeces

Crucible or Foil no. & animal nos.	Initial wt. of foil (mg)	Dry wt. of sample + foil (mg)	Dry wt of sample only (mg)	Dry wt. after blank correction (mg)	Ash wt. sample + foil (mg)	Ash wt of sample only (mg)	Ash wt. after blank correction (mg)	E value	Absorption efficiency
11/ 1 to 4	12755.37	12761.76	6.39	5.99	12756.66	1.29	0.92	0.846	0.452
12/ 5 to 8	12229.86	12236.21	6.35	5.95	12231.22	1.36	0.99	0.834	0.488
13/ 9 to 12	10138.53	10145.85	7.32	6.92	10139.87	1.34	0.97	0.860	0.407
14/ 13 to 16	11707.46	11714.50	7.04	6.64	11708.84	1.38	1.01	0.848	0.447
								Mean=	**0.449**
								N=	**4**
								S.E.=	**0.017**

Note that crucibles were used in this example, but aluminium foil containers are now used.

Annex 6: Table of oxygen solubilities (μmoles O_2 l^{-1}) with temperature and salinity

	SALINITY														
Temp °C	**0**	**10**	**15**	**25**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**
0	457.0	425.9	411.0	382.9	369.5	366.9	364.3	361.7	359.2	356.6	354.1	351.6	349.1	346.6	344.2
5	399.0	373.3	361.1	337.7	326.6	324.4	322.3	320.1	318.0	315.9	313.8	311.7	309.6	307.6	305.5
10	352.8	331.2	320.9	301.2	291.8	289.9	288.1	286.3	284.4	282.6	280.9	279.1	277.3	275.6	273.8
11	344.7	323.8	313.8	294.7	285.5	283.8	282.0	280.2	278.5	276.7	275.0	273.3	271.5	269.8	268.1
12	336.9	316.6	306.9	288.4	279.6	277.8	276.1	274.4	272.7	271.0	269.3	267.6	266.0	264.3	262.7
13	329.4	309.8	300.3	282.4	273.8	272.1	270.4	268.7	267.1	265.4	263.8	262.2	260.6	259.0	257.4
14	322.2	303.1	294.0	276.5	268.2	266.6	264.9	263.3	261.7	260.1	258.5	256.9	255.4	253.8	252.3
15	315.3	296.8	287.9	270.9	262.8	261.2	259.6	258.0	256.5	254.9	253.4	251.8	250.3	248.8	247.3
16	308.6	290.6	282.0	265.5	257.6	256.0	254.5	253.0	251.4	249.9	248.4	246.9	245.4	244.0	242.5
17	302.2	284.7	276.3	260.2	252.5	251.0	249.5	248.0	246.5	245.1	243.6	242.2	240.7	239.3	237.8
18	296.0	278.9	270.8	255.1	247.6	246.2	244.7	243.3	241.8	240.4	239.0	237.5	236.1	234.7	233.3
19	290.0	273.4	265.4	250.2	242.9	241.5	240.1	238.6	237.2	235.8	234.4	233.1	231.7	230.3	229.0
20	284.3	268.1	260.3	245.4	238.3	236.9	235.5	234.2	232.8	231.4	230.1	228.7	227.4	226.0	224.7
21	278.7	262.9	255.3	240.8	233.9	232.5	231.2	229.8	228.5	227.1	225.8	224.5	223.2	221.9	220.6
22	273.3	257.9	250.5	236.3	229.6	228.2	226.9	225.6	224.3	223.0	221.7	220.4	219.1	217.8	216.6
23	268.1	253.1	245.8	232.0	225.4	224.1	222.8	221.5	220.2	218.9	217.7	216.4	215.2	213.9	212.7
24	263.1	248.4	241.3	227.8	221.3	220.0	218.8	217.5	216.2	215.0	213.8	212.5	211.3	210.1	208.9
25	258.2	243.8	236.9	223.7	217.3	216.1	214.9	213.6	212.4	211.2	210.0	208.8	207.6	206.4	205.2
26	253.5	239.4	232.7	219.7	213.5	212.3	211.1	209.9	208.7	207.5	206.3	205.1	203.9	202.8	201.6
27	249.0	235.2	228.5	215.8	209.8	208.6	207.4	206.2	205.0	203.9	202.7	201.5	200.4	199.2	198.1
28	244.5	231.0	224.5	212.1	206.1	205.0	203.8	202.6	201.5	200.3	199.2	198.1	196.9	195.8	194.7
29	240.3	227.0	220.6	208.4	202.6	201.4	200.3	199.2	198.0	196.9	195.8	194.7	193.6	192.5	191.4
30	236.1	223.1	216.9	204.9	199.1	198.0	196.9	195.8	194.7	193.6	192.5	191.4	190.3	189.2	188.1
31	232.1	219.3	213.2	201.4	195.8	194.7	193.6	192.5	191.4	190.3	189.2	188.2	187.1	186.0	185.0
32	228.2	215.6	209.6	198.1	192.5	191.4	190.3	189.3	188.2	187.1	186.1	185.0	184.0	182.9	181.9
33	224.4	212.1	206.1	194.8	189.3	188.3	187.2	186.1	185.1	184.0	183.0	182.0	180.9	179.9	178.9
34	220.7	208.6	202.7	191.6	186.2	185.2	184.1	183.1	182.0	181	180.0	179.0	178.0	176.9	175.9
35	217.1	205.2	199.5	188.4	183.2	182.1	181.1	180.1	179.1	178.1	177.0	176.0	175.0	174.1	173.1
36	213.7	201.9	196.2	185.4	180.2	179.2	178.2	177.2	176.2	175.2	174.2	173.2	172.2	171.2	170.3
37	210.3	198.7	193.1	182.4	177.3	176.3	175.3	174.3	173.3	172.4	171.4	170.4	169.4	168.5	167.5
38	207.0	195.6	190.1	179.5	174.5	173.5	172.5	171.5	170.6	169.6	168.6	167.7	166.7	165.8	164.8
39	203.8	192.5	187.1	176.7	171.7	170.8	169.8	168.8	167.9	166.9	166.0	165.0	164.1	163.1	162.2
40	200.7	189.6	184.2	174.0	169.1	168.1	167.1	166.2	165.2	164.3	163.3	162.4	161.5	160.6	159.6

Annex 7: Example of spreadsheet for calculating respiration rate

Calculation of Oxygen Consumption

Experiment: ***Wash Study.***
Sample: ***Site H.***
Date of expt.: ***August 1995.***

O2 Sat. umol O2/L =		**259**					
Standard weight(g)=		**1**		**Weight exponent =**			**0.67**
Ani.	Dry wt. (g)	Resp. vol (L)	Start (mmHg)	End (mmHg)	Time (min)	O2 uptake (umol/h)	O2 uptake (umol/h/g)
1	0.464	0.519	143.8	138.2	40	7.15	11.95
2	0.462	0.526	147.9	141.4	40	8.41	14.10
3	0.394	0.550	150.3	144.3	40	8.11	15.14
4	0.471	0.524	145.7	139.4	40	8.12	13.44
5	0.462	0.528	153.1	146.5	40	8.57	14.37
6	0.476	0.468	151.0	143.0	40	9.21	15.14
7	0.336	0.536	149.8	143.8	40	7.91	16.42
8	0.482	0.502	144.3	136.9	40	9.13	14.89
9	0.418	0.519	151.6	145.8	40	7.40	13.28
10	0.467	0.526	142.3	137.5	40	6.21	10.34
11	0.454	0.550	143.9	137.0	40	9.33	15.84
12	0.399	0.524	144.9	139.5	40	6.96	12.88
13	0.504	0.528	147.2	140.9	40	8.18	12.94
14	0.485	0.468	146.9	141.8	40	5.87	9.53
15	0.438	0.536	145.5	137.9	40	10.02	17.42
16	0.404	0.502	143.3	134.3	40	11.11	20.39
				Mean=		**8.23**	**14.26**
				N=		**16**	**16**
				S.E.=		**0.34**	**0.66**
				Upper 95% CI =		**8.91**	**15.58**
				Lower 95% CI =		**7.55**	**12.93**

Annex 8: Correction for body size

Body size is an important variable affecting most physiological responses, but one that can be largely eliminated by selecting and transplanting animals of similar body size. It is inevitable, however, that there will be slight differences in the dry body mass, and this effect can be removed by correcting rates of feeding, respiration, excretion, and growth to a standard body size using the allometric equation:

$$(1)\quad Y = aX^b$$

or $$(2)\quad \log Y = \log a + b \log X$$

where Y = physiological rate, X = dry body mass (g), and a and b are the intercept and slope, respectively. Physiological rates are converted to an appropriate weight-specific rate using the exponent b.

The equations describing the relationships between each physiological rate and dry body mass are first established for a baseline reference population. Approximately 30 individuals covering a wide size range are measured, and the data are then analysed by linear regression of log-transformed data (X, Y). The weight exponent or slope of the regression for each physiological response is then used to correct for differences in dry body mass found within any sample. If animals of approximately 1 g dry mass are selected and measured, rates can be corrected to a "standard 1 g animal".

For example:

The slope (b = 0.67) describing the relationship between oxygen uptake and dry body mass is substituted in equation 2. Therefore, if an individual has an oxygen uptake of 12.54 µmoles O_2 h^{-1} and a dry mass of 0.83 g then:

$$\log a = \log Y - b \log X$$

$$\log a = \log 12.54 - 0.65 (\log 0.83)$$

$$a = 14.21 \text{ µmoles } O_2 \text{ g}^{-1} \text{ h}^{-1}$$

If the animals' average body mass is markedly different from 1 g dry mass, then a standard body size equivalent to the mean body mass is chosen, and the corrections for any weight differences are made in a similar manner, but using the following equation:

$$\log Y_c = \log Y_o - (b \log X_o - b \log X_c)$$

where Y_c is the corrected value for a standard body mass (X_c), and Y_o and X_o are the individual's measured rate and body mass, respectively.

Annex 9: Example of a spreadsheet for calculating SFG

Calculation of Scope For Growth

Experiment: ***Wash Study.***
Sample: ***Site H.***
Date: ***August 1995.***

Wt. exponent = 0.67 Standard weight = 1 Absorp. effcy. = 0.45
Food energy value = 10 Joules/ litre.

An.	D Wt (g)	Length (mm)	C.R. (l/h)	C.R. (l/h/g)	C (J/h/g)	A (J/h/g)	Resp. (umol/h/g)	R (J/h/g)	SFG (J/h/g)
1	0.46	41.13	3.47	5.81	58.12	26.15	12.23	5.58	20.58
2	0.46	41.91	2.22	3.73	37.28	16.78	14.43	6.58	10.20
3	0.39	40.60	1.75	3.27	32.73	14.73	15.57	7.10	7.63
4	0.47	39.61	3.04	5.03	50.35	22.66	13.75	6.27	16.39
5	0.46	40.75	3.96	6.64	66.38	29.87	14.61	6.66	23.21
6	0.48	41.42	3.66	6.02	60.23	27.11	15.48	7.06	20.05
7	0.34	41.87	2.64	5.48	54.83	24.67	16.97	7.74	16.94
8	0.48	40.35	2.78	4.54	45.37	20.42	15.22	6.94	13.48
9	0.42	40.09	3.16	5.66	56.63	25.48	13.63	6.22	19.27
19	0.47	40.14	3.03	5.05	50.52	22.73	10.58	4.82	17.91
11	0.45	41.15	2.66	4.52	45.17	20.33	16.22	7.40	12.93
12	0.40	40.40	2.29	4.24	42.40	19.08	13.24	6.04	13.04
13	0.50	41.00	2.66	4.21	42.11	18.95	13.21	6.03	12.92
14	0.49	40.35	3.18	5.16	51.64	23.24	9.74	4.44	18.80
20	0.44	41.91	3.25	5.65	56.51	25.43	17.85	8.14	17.29
16	0.40	41.29	2.93	5.37	53.70	24.17	20.95	9.55	14.61
Mean	0.44	40.87	2.92	5.02	50.25	22.61	14.61	6.66	**15.95**
SE	0.01	0.18	0.14	0.22	2.23	1.00	0.68	0.31	**1.03**
2 S.E.	0.02	0.35	0.28	0.45	4.45	2.00	1.37	0.62	**2.06**
L 95% C.I.	0.42	40.52	2.64	4.58	45.80	20.61	13.24	6.04	**13.89**
U 95% C.I.	0.47	41.22	3.20	5.47	54.70	24.61	15.97	7.28	**18.01**
N	16	16	16	16	16	16	16	16	**16**

ICES Techniques in Marine Environmental Sciences

No. 1 Cadmium and lead: Determination in organic matrices with electrothermal furnace atomic absorption spectrophotometry

No. 2 Trace metals in seawater: Sampling and storage methods

No. 3 Cadmium in marine sediments: Determination by graphite furnace atomic absorption spectroscopy

No. 4 Lipophilic organic material: An apparatus for extracting solids used for their concentration from seawater

No. 5 Primary production: Guidelines for measurement by ^{14}C incorporation

No. 6 Control procedures: Good laboratory practice and quality assurance

No. 7 Suspended particulate matter: Collection methods for gravimetric and trace metal analysis

No. 8 Soft bottom macrofauna: Collection and treatment of samples

No. 9 Sediments and suspended particulate matter: Total and partial methods of digestion (*videotape available*)

No. 10 Organic halogens: Determination in marine media of adsorbable, volatile, or extractable compound totals

No. 11 Biological effects of contaminants: Oyster (*Crassostrea gigas*) embryo bioassay

No. 12 Hydrocarbons: Review of methods for analysis in seawater, biota, and sediments

No. 13 Biological effects of contaminants: Microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish

No. 14 Temporal trend monitoring: Introduction to the study of contaminant levels in marine biota

No. 15 Temporal trend monitoring: Contaminant levels in tissues of Atlantic cod

No. 16 Benthic communities: Use in monitoring point-source discharges

No. 17 Nutrients: Practical notes on their determination in seawater

No. 18 Contaminants in marine organisms: Pooling strategies for monitoring mean concentrations

No. 19 Common diseases and parasites of fish in the North Atlantic: Training guide for identification

No. 20 Temporal trend monitoring: Robust method for analysing contaminant trend monitoring data

No. 21 Chlorobiphenyls in marine sediments: Guidelines for determination

No. 22 Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds

No. 23 Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity

No. 24 Biological effects of contaminants: Use of imposex in the dogwhelk (*Nucella lapillus*) as a bio-indicator of tributyltin pollution

No. 25 Biological effects of contaminants: Measurement of DNA adducts in fish by ^{32}P-postlabelling

No. 26 Biological effects of contaminants: Quantification of metallothionein (MT) in fish liver tissue

No. 27 Soft bottom macrofauna: Collection, treatment, and quality assurance of samples

No. 28 Biological effects of contaminants: *Corophium* sp. sediment bioassay and toxicity test

No. 29 Biological effects of contaminants: Sediment bioassay using the polychaete *Arenicola marina*

No. 30 Chlorophyll *a*: Determination by spectroscopic methods

No. 31 Biological effects of contaminants: Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins

No. 32 Biological monitoring: General guidelines for quality assurance

No. 33 Recruitment studies: Manual on precision and accuracy of tools

No. 34 Biological effects of contaminants: Quantification of d-aminolevulinic acid dehydratase (ALA-D) activity in fish blood

No. 35 Chemical measurements in the Baltic Sea: Guidelines on quality assurance

No. 36 Biological effects of contaminants: Measurement of lysosomal membrane stability

No. 37 Biological effects of contaminants: Use of intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution

No. 38 Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus*

No. 39 Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile

No. 40 Biological effects of contaminants: Measurement of scope for growth in mussels