Key words: Zooplankton, Respiration, Excretion, ETS, GDH

10 Metabolism

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Review

10.1 OXYGEN CONSUMPTION AS AN INDEX OF METABOLISM

'Metabolism' may be defined as the sum of all processes through which protoplasm is formed from food (anabolism) and broken down into waste matter (catabolism), with release of energy. Energy gained through the processes is stored exclusively in the form of adenosine triphosphate (ATP), and used for various activities (locomotion, production of new tissue, ion pumps etc.) of living organisms. ATP is generated from the tricarboxylic-acid (TCA) cycle and oxygen is required to drive the cycle. Therefore metabolic rate may be estimated by measuring oxygen consumption rate. Exceptions are anaerobiosis in intertidal organisms and burst escape activity, but the oxygen debt incurred is usually repaid later when oxygen is once more available (cf. Clarke 1987). 'External respiration' is the mechanism by which oxygen is brought into and carbon dioxide expelled from the organism, whereas 'internal respiration' refers to the sum of enzymatic reactions, both oxidative and nonoxidative, by which energy is made available for biological work (Prosser 1961). In most ecological studies dealing with intact organisms, 'external respiration' is of special interest and is simply referred as 'respiration'. For this reason, oxygen consumption rate is referred to as respiration (or respiratory) rate or *vice versa*. In this sense, either oxygen consumption or carbon dioxide production can be used as an index of metabolic rate of marine zooplankton. However, accurate determination of carbon dioxide in sea water is hampered by the strong buffering action of sea water and has been used in only a few studies, i.e. *Neomysis integer* by Raymont and Krishnaswamy (1968) and *Rhincalanus gigas* by Rakusa-Suszczewski *et al.* (1976). In contrast, accurate determination of dissolved oxygen in sea water became possible with the advent of the Winkler titration method in the late 1800s (Winkler 1888). In the early 1960s, oxygen-electrodes were introduced to the study of marine zooplankton metabolism as a simple tool for measuring dissolved oxygen in sea water (Teal and Halcrow 1962; Halcrow 1963).

Examples of very early studies on the oxygen consumption rates of marine zooplankton may be found in the work of Vernon (1896) on salps, followed by that of Ostenfield (1913, cited in Marshall 1973) on the copepod *Calanus hyperboreus*. Since then voluminous data have been accumulated on the metabolism of various zooplankton species living in many regions of the world's oceans (for review, see Marshall 1973; Ikeda 1974; Conover 1978; Raymont 1983). Part of the data on selected zooplankton species are shown in Table 10.1. Omori and Ikeda (1984) and Le Borgne (1986) reviewed methodologies for studies of marine zooplankton metabolism.

The metabolic rate of animals is defined with respect to the activity of animals as follows: 'standard' (or 'basal') metabolism is the oxygen consumption rate for main-

ICES Zooplankton Methodology Manual
ISBN 0-12-327645-4

Key words: Zooplankton, Respiration, Excretion, ETS, GDH

Table 10.1 Rates of oxygen consumption, ammonia excretion and inorganic phosphate excretion of various marine zooplankton species determined by incubating specimens in filtered sea water. — = no data, indiv = individual.

Zooplankton species	Location	Experimental temperature (°C)	DW (mg)	Oxygen consumption ($\mu l\ O_2\ indiv^{-1}\ h^{-1}$)	Ammonia excretion ($\mu gN\ indiv^{-1}\ h^{-1}$)	Phosphate excretion ($\mu gP\ indiv^{-1}\ h^{-1}$)	Source
Coelenterata							
Aglantha digitale	Barents Sea	1.1	14.0	2.27	0.082	0.035	Ikeda and Skjoldal (1989)
Ctenophora							
Mnemiopsis leidyi	Narragansett Bay	20	299	30.35	2.93	1.00	Kremer (1977)
Thecosomata							
Cavolinia longirostris	GBR inshore water	27	8.9	16.7	1.81	0.30	Ikeda and Skjoldal (unpubl.)
Limacina helicina	Barents Sea	0.0	0.593	0.451	0.0072	0.0052	Ikeda and Skjoldal (1989)
Gymnosomata							
Clione limacina	Barents Sea	−0.1	16.47	1.65	0.034	0.021	Ikeda and Skjoldal (1989)
Copepoda							
Calanus finmarchicus, C6♀	Barents Sea	0.1	0.387	0.328	0.013	0.0050	Ikeda and Skjoldal (1989)
C. glacialis, C6♀	Barents Sea	3.5	0.410	0.630	0.026	0.010	Ikeda and Skjoldal (1989)
C. hyperboreus, C6♀	Barents Sea	1.3	3.95	1.49	0.049	0.0050	Ikeda and Skjoldal (1989)
Neocalanus cristatus, C5	off Hokkaido	6.3	1.59	1.67	0.164	—	Ikeda (1974)
N. plumchrus, C5	off Hokkaido	7.3	0.785	0.68	0.017	—	Ikeda (1974)
Eucalanus bungii, C6♀	off Hokkaido	6.0	1.01	0.80	0.058	—	Ikeda (1974)
Metridia pacifica, C6♀	off Hokkaido	7.7	0.182	0.383	0.019	—	Ikeda (1974)
Acartia australis	GBR inshore water	24.5	0.0091	0.056	0.0054	0.0018	Ikeda and Skjoldal (1980)
Undinula darwini	Indian Ocean	28.5	0.15	1.13	0.137	0.044	Gaudy and Boucher (1983)
Euchaeta marina	Indian Ocean	28.5	0.22	1.30	0.131	0.052	Gaudy and Boucher (1983)
Temora discaudata	Indian Ocean	28.5	0.034	0.23	0.020	0.010	Gaudy and Boucher (1983)

Mysidacea							
Acanthomysis pseudomacropsis♀	off Hokkaido	14.9	2.88	4.36	0.630	—	Ikeda (1974)
Amphipoda							
Themisto libellula	Barents Sea	−0.1	2.98	2.53	0.053	0.018	Ikeda and Skjoldal (1989)
Euphausiacea							
Thysanoessa inermis	Barents Sea	1.9	34.78	13.77	0.855	0.33	Ikeda and Skjoldal (1989)
Euphausia pacifica	off Hokkaido	10.2	12.95	16.10	0.781	—	Ikeda (1974)
E. lucens	off SW Africa	12.5	5.00	10.47	0.850	—	Stuart (1986)
Chaetognatha							
Sagitta elegans	Barents Sea	−0.3	4.50	1.41	0.345	0.028	Ikeda and Skjoldal (1989)
S. enflata	GBR inshore water	25	0.71	1.67	0.12	0.021	Ikeda and Skjoldal (unpubl.)
Appendiculata							
Oikopleura dioica	Lab culture	24	0.0032	0.209	0.0125	0.0012	Gorsky *et al.* (1987)
Thaliacea							
Salpa thompsoni, sol.	Antarctic water	−1.0	114.63	12.0	0.806	0.39	Ikeda and Mitchell (1982)
Thalia democratica, sol.	off S. Africa	17.3	2.97	1.77	0.18	—	Ikeda (1974)

tenance only, 'routine' metabolism is the oxygen consumption rate measured with uncontrolled but minimum motor activity, and 'active' metabolism is the oxygen consumption rate with enforced activity at a maximal level. When the oxygen consumption rate is measured at different activity levels, the rate extrapolated to zero activity is the standard metabolism. In most studies on zooplankton metabolism, no attempts have been made to relate the oxygen consumption data to the level of activity of animals on the premise that the measured rates are close to routine metabolism. As notable exceptions, Torres and Childress (1983) and Buskey (1998) established the relationship between oxygen consumption rates and swimming speed in the migrating euphausiid *Euphausia pacifica* and swarming copepod *Dioithona oculata* respectively. According to their results, standard metabolism is 0.7 and active metabolism is 2.7 times routine metabolism in *E. pacifica*, and respective figures are 0.5 and 3.3 times routine metabolism in *D. oculata*. As an alternative approach, net cost of swimming for several crustacean plankton has been calculated on the theoretical grounds of mechanical power dissipated as drag (cf. Morris *et al.* 1990, and references therein).

10.1.1 Conversion of oxygen consumption to carbon and calorific units

The molar ratio of carbon dioxide produced to oxygen consumed is called the 'Respiratory Quotient' (*RQ*). In theory, the *RQ* varies from 0.71 to 1.0 depending on metabolic substrates (lipid, 0.71; protein, 0.80; carbohydrate, 1.0; from Prosser 1961). According to Gnaiger's (1983) re-calculation the *RQ* value for carbohydrate remains unchanged (1.0), but for lipid, it is 0.72 and for protein it changes as a function of the excretory end-product, i.e. 0.97 for ammonia and 0.84 for urea. Since marine zooplankton are considered to be primarily ammonotelic (see Nitrogen and phosphorous metabolism in section 10.2) an *RQ* of 0.97 may be more appropriate to convert their oxygen consumption data to carbon units;

$$\text{ml } O_2 \text{ (individual h)}^{-1} \times RQ \times 12/22.4 = \text{mg C (individual h)}^{-1} \quad (10.1)$$

where 12/22.4 is the weight (12 g) of carbon in 1 mole of (22.4 l) of carbon dioxide. Oxygen consumption rate expressed in units of carbon represents the carbon requirement for zooplankton metabolism, and can be used as an index of minimum food requirements when assimilation efficiency and growth are not taken into account.

The caloric equivalent to a unit volume of oxygen consumed varies depending on the metabolic substrate utilized. According to Gnaiger (1983), there are 440, 447 and 471 kilo joules (kJ) per mole of oxygen consumed for lipid, protein (ammonia as end-product) and carbohydrate, respectively. Since 1 mole of oxygen is 22.4 l and 1 calorie is equivalent to 4.1868 J, the oxycaloric equivalents can be expressed in calories as 4.69, 4.76 and 5.02 kcal per liter of oxygen consumed for lipid, protein and carbohydrate metabolism, respectively.

10.2 NITROGEN AND PHOSPHORUS METABOLISM

Animals produce various substances as end-products of metabolism. Although zooplankton excreta include both liquid and solid forms (fecal pellets) we will only consider here liquid forms. Nitrogen compounds have been measured in terms of total N, ammonia-N, amino-N and urea-N, and phosphorus compounds in terms of total-P, inorganic-P and organic-P. Among these, ammonia and inorganic phosphorus are of

special interest because of their importance as immediately available nutrients for phytoplankton (cf. Corner and Davies 1971). Excretion rates of ammonia and inorganic phosphate of selected zooplankton species are shown in Table 10.1.

A major end-product of nitrogen metabolism in aquatic animals is ammonia, in contrast to urea or uric acids in terrestrial animals (Wright 1995). Ammonia is present in two forms in sea water: unionized ammonia (NH_3) and ammonium ion (NH_4^+). The form of ammonia excreted by zooplankton is not certain (for crustaceans see Regnault 1987). The forms of ammonia in sea water may be expressed by the equilibrium equation:

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad (10.2)$$

The equilibrium reaction is influenced by temperature and pH. In the usual pH range (7.5–8.2) of sea water and at 18 °C, less than 5% of ammonia is in the form of NH_3. This percentage increases as temperature or pH increases. Because of the lack of information about ammonia forms excreted by marine zooplankton and complex equilibrium of ammonia in sea water, ammonia excreted by marine zooplankton has been referred as either ammonia (NH_3) or ammonium (NH_4^+) (note that NH_3-N and NH_4^+-N are comparable to each other in terms of N). In the following no distinction is made to between the two forms of ammonia.

Ammonia is well documented as the major form of dissolved nitrogen excreted by marine zooplankton. For *Neomysis rayii* and *Euphausia pacifica*, ammonia-N accounted for 75%–85% of the total-N excreted, followed by 10%–24% as amino-N and 0%–1% as urea-N (Jawed 1969). Ikeda and Skjoldal (1989) determined excretion rates of ammonia-N, urea-N and dissolved free amino acids (DFAA)-N of five zooplankton species (*Clione limacina, Calanus glacialis, C. hyperboreus, Themisto libellula* and *Sagitta elegans*) and noted that ammonia-N was the most important (78%–93% of the total), followed by urea-N (1%–23%) and DFAA-N (6%–25%). Corner and Newell (1967) reported that 60%–100% of the total-N excreted by *Calanus helgolandicus* was ammonia and the rest was urea. Corner *et al.* (1976) confirmed this result for the same species fed barnacle nauplii; only 9%–10% of total-N excreted was urea. Ammonia is also the dominant form of nitrogen excreted by the ctenophore *Mnemiopsis leidyi* (Kremer 1977). While all these studies were made using the sealed chamber method mentioned below (Measuring metabolic rate on live zooplankton, section 10.3), Gardner and Paffenhofer (1982) determined ammonia and amino-N excretion by *Eucalanus pileatus* every 10 minutes with a flow-through system (see measuring metabolic rate on live zooplankton, section 10.3). According to Gardner and Paffenhofer's results, the amount of nitrogen excreted in the form of ammonia was far greater than that excreted as amino-N for this copepod. The time course of amino-N excretion was characterized by a discontinuous pattern. Although the mechanism of amino-acid release is not clear, it may come from food during feeding ('sloppy feeding'), from diffusion across cell membranes, from catabolism of food, or from osmoregulation (Gardner and Paffenhofer 1982; Forward and Fyhn 1983; Williams and Poulet 1986).

In contrast to studies showing ammonia as the major nitrogenous excretory product, Webb and Johannes (1967) reported abnormally high excretion of dissolved free amino-acids by mixed zooplankton. The results of Webb and Johannes were considered to be an artifact caused by an extremely high density of zooplankton in their experiments, i.e. amino-N leaking from the injured specimens and possible cannibalism during incubation, although Webb and Johannes (1969) suggested that the contradictory results of other workers were due to overlooking the bacterial uptake of amino-acids during the incubation (see page 471).

Dissolved phosphorus compounds in zooplankton excreta can be separated into inorganic and organic fractions (technically, the organic fraction is not measured directly but calculated from the difference between the total and inorganic fractions). Pomeroy *et al.* (1963) reported that 33%–55% of the total-P excreted by mixed zooplankton was inorganic. Later, Hargrave and Geen (1968) found a high percentage in the organic fraction (up to 74% of the total-P excreted) for three copepod species. According to the seasonal study of Butler *et al.* (1969, 1970), the percentage of the organic fraction in the total-P excreted by *Calanus finmarchicus* was highest (70%) in spring when phytoplankton food was most abundant, but the inorganic fraction dominated during the food-depleted winter. It should be noted that this seasonal change in the relative amounts of organic and inorganic-P was not seen for nitrogen excretion, in which ammonia comprised a constant 88% of the total-N excreted by the copepod. For *Euphausia superba* maintained with and without food for 7 months, Ikeda and Dixon (1982) found that while both organic and inorganic fractions of both nitrogen and phosphorus excreta were determined in fed specimens, only the inorganic fraction was detected for starved specimens.

As was the case for nitrogen excretion, discrepancies seen in the results of phosphorus excretion experiments by previous workers may be caused by dissimilar experimental designs including the use of individual or mixed species (see page 462). Feeding conditions of zooplankton prior to or during the experiments may be considered prime factors influencing the proportion of organic fraction in total phosphorus excretion (see page 467).

10.3 MEASURING METABOLIC RATE ON LIVE ZOOPLANKTON

Determination of metabolic rate is straightforward in theory and rather simple in practice. However, a number of experimental conditions are known to affect the results. Techniques currently in use for determining oxygen consumption and excretion rates of ammonia and phosphorus on live zooplankton can be classified largely into two types.

1) Sealed chamber method Specimens are confined in containers filled with sea water for a certain period and the decrease of oxygen or increase of excretory products during the period (several hours to a day) are monitored throughout, or determined at the end of the incubation. Despite the possible detrimental effects of oxygen depletion and accumulation of excreta, the method is simple in practice and has been used extensively. The technical problems associated largely with this method are reviewed in detail in the following section (Technical problems, section 10.3.1).
2) Flow-through method Oxygen consumption and accumulation of excreta are measured continuously on the specimens placed in a flow-through system. This method avoids some of the problems associated with method (1) but it often requires that specimens be placed in a very small cell. In addition, it requires a rigorous calculation taking into consideration flow-rate, cell size and metabolic rates of zooplankton to obtain reliable data (Northby 1976; Niimi 1978; Propp *et al.* 1982). Application of this method has been limited to the study of Gerber and Gerber (1979) for simultaneous measurements of ingestion and oxygen consumption rate of tropical lagoon zooplankton, that of Gardner and Scavia (1981) for nitrogen excretion of *Daphnia* sp., and that of Gardner and Paffenhofer (1982) for nitrogen excretion of *Eucalanus pileatus*.

10.3.1 Technical problems

In the following, various sources of potential error in measuring metabolic rates of zooplankton collected from the field are reviewed and discussed. For this purpose, only key references were selected. While most of the references concern zooplankton, benthic species and fishes are also referred to when appropriate.

CAPTURE STRESS/STARVATION

A common problem with experimental studies of zooplankton is the delay between actual capture of specimens and subsequent measurements. Such delays may range from several hours to several tens of hours; they are often unavoidable due to the logistics of field sampling and laboratory routines. Some studies have noted decreases in metabolic rates of zooplankton with the increase of time after capture, and a question naturally arises as to what causes this. The validity of measurements taken at varying times after capture may be called into question as well.

The first observation of this nature was made by Marshall *et al.* (1935), who reported a rapid fall in the oxygen consumption rate of *Calanus finmarchicus* soon after capture. Since then, similar results have confirmed this phenomenon, not only in oxygen consumption rate, but also in the excretion rates of ammonia and inorganic phosphorus of several zooplankton species (Ikeda and Skjoldal 1980; Skjoldal *et al.* 1984 and references therein). Compared with that of oxygen consumption rate, ammonia and phosphorus excretion rates in zooplankton just after capture are more variable due to the switch in metabolic substrates (e.g. from protein to lipid, cf. Skjoldal *et al.* 1984).

Stress incurred during the course of sampling and possible starvation of specimens in captivity may be considered as part of the cause of the rapid fall in metabolic rates after capture (Figure 10.1). The magnitude of both effects on metabolic rates may vary from species to species, depending on the depth of occurrence of zooplankters, the sampling gear used, weight-specific metabolic rates, temperature, etc. From this scheme, a higher oxygen consumption rate just after capture may be explained by 'oxygen debt' brought about by the extra exercise during capture; this capture stress would be greatest at the beginning of an experiment. It is difficult to provide the quantity and quality of appropriate food during experiments, thereby the effect of starvation predominates during the progress of experiments.

Capture stress has been effectively demonstrated in the oxygen consumption measurements of sockeye salmon (*Oncorhynchus nerka*) by Brett (1964). Most of the energy needed during heavy extra exercise of sockeye salmon is supplied by anaerobic processes, not through the TCA-cycle; this is demonstrated by excessive accumulation of lactic acid in the blood (Black *et al.* 1962, 1966). For zooplankton, adenylate energy charge (EC) ratio has been used as an index of capture stress for the initial decreases in metabolic rate of freshly caught specimens of the copepod *Acartia australis*, the decapod shrimp *Acetes sibogae australis* (Ikeda and Skjoldal 1980), and the copepod *Euchaeta norvegica* (Skjoldal *et al.* 1984). However, no appreciable capture stress was detected from the results of this index in both studies. In addition, lactic acid levels in *E. norvegica* just after capture were low and not indicative of any oxygen debt (Skjoldal *et al.* 1984). Thus, the results of Ikeda and Skjoldal (1980) and Skjoldal *et al.* (1984) indicate that starvation was the primary cause for the decrease in metabolic rates of these zooplankters.

A progressive decrease in metabolic rates of starved specimens for hours, days or weeks has been demonstrated in various zooplankton species (Conover 1968; Mayzaud 1976; Ikeda 1977a, 1977b; Båmstedt and Tande 1985; Kremer and Reeve 1989). The

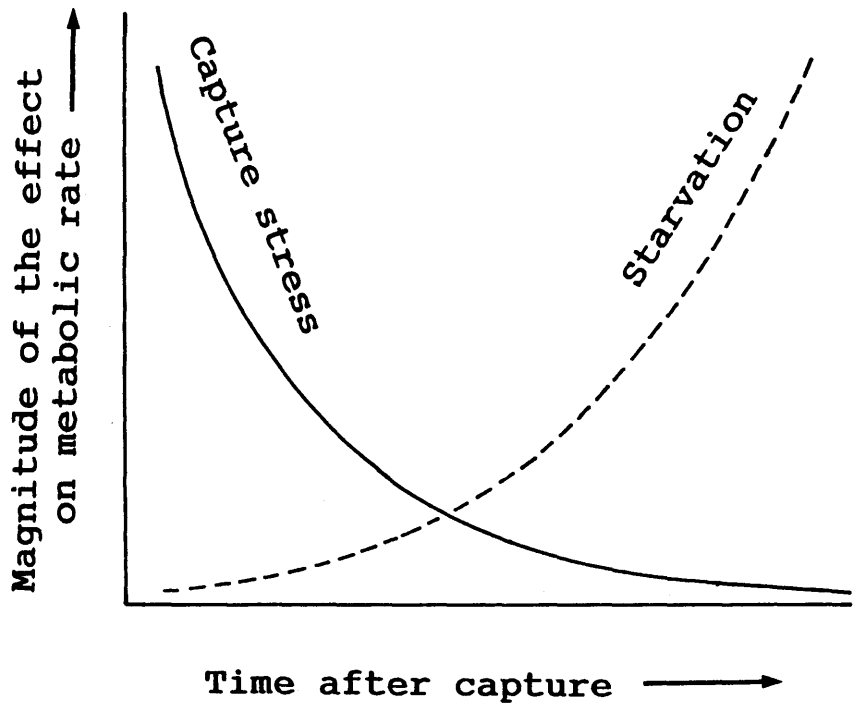

Fig. 10.1 Schematic presentation of the sequential changes of the effects of capture stress and starvation on the metabolic rate of zooplankton after capture.

effect of starvation can be minimized by the use of zooplankton immediately after capture, provided there is no capture stress. Provision of appropriate food during the time between capture and experimental manipulation may be an alternative, but prolonged maintenance of wild zooplankton in the laboratory may change its behavior, swimming activity, and nutritional condition, all of which could affect the metabolic rates.

Biggs (1977) and Cetta *et al.* (1986) enclosed gelatinous zooplankton directly in glass jars while Scuba-diving for the measurements of oxygen consumption and ammonia excretion rates. For oxygen consumption measurements Smith (1982), Youngbluth *et al.* (1988) and Bailey *et al.* (1994) adopted a gentle suction of animals into a chamber equipped with an oxygen sensor and incubated animals in *in situ* conditions on board submersibles. All these procedures are considered to be superior to that of the conventional catch–transfer–incubation method (i.e. sealed chamber method) to minimize the effects of handling and laboratory conditions. However, application of these methods is costly and it is difficult to control contamination by bacteria and microzooplankton in the incubation containers. Presently, *in situ* methods have been limited to large zooplankton which are recognizable by the unaided eye.

CONTAINER SIZE/CROWDING

The use of smaller containers or high densities of specimens may be needed to bring a measurable difference in oxygen and/or nutrient concentrations between experimental and control chambers. Marshall and Orr (1958) examined the effect of container size on the oxygen consumption rate of *Calanus finmarchicus* using various container sizes (30–

160 ml) and found no appreciable differences between the results. Zeiss (1963) compared oxygen consumption rates of *Calanus finmarchicus* and *Daphnia magna* placed under several densities of specimens. While the rates of *C. finmarchicus* were not affected in the range of 2.5 to 10 specimens ml^{-1}, the rate of *D. magna* increased at higher densities. Razouls (1972) measured the oxygen consumption rate of *Temora stylifera* and *Centropages typicus* at densities from 0.1 to 2 individuals ml^{-1}. The highest oxygen consumption rates were obtained at 0.5 individual ml^{-1} and these rates decreased at lower and higher densities in both species.

Some density effects have been reported in the excretion rates of nutrients by zooplankton. In *Acartia tonsa* the phosphorus excretion rate decreased when the density of individuals exceeded 400 specimens ml^{-1} (Hargrave and Geen 1968). In contrast, Nival *et al.* (1974) obtained anomalously high ammonia excretion rates for *Temora stylifera* incubated at densities greater than 200 specimens ml^{-1}.

Container size and crowding are analogous in terms of volume available per specimen, i.e. density, but the former is largely interaction with container wall and the latter interaction between individuals. Therefore, the effect of these parameters on metabolic rates could vary depending on species-specific differences in swimming behavior, perception of stimuli, tolerance to a rapid depletion of oxygen, and associated accumulation of excreta. For carnivorous species, cannibalism could easily be induced by the increase in density. Thus, metabolic rates of zooplankton could be either enhanced or depressed by higher densities as described above. As a general guideline for designing metabolic studies, experiments using higher densities of specimens should be avoided to obtain the best results. Information about natural density of test zooplankton species should be useful to design experimental densities of specimens for their metabolic measurements, and check the validity of the results whenever necessary.

INJURY/DEATH

Physical injury of specimens (such as broken legs or rupture of a part of the body) incurred during collection at sea or handling in the laboratory prior to their use for experiments can easily happen, but is often difficult to eliminate, especially for small zooplankton. Including injured or dead specimens during incubation are possible sources of error in determining metabolic rates of zooplankton. Mullin *et al.* (1975) found that both ammonia and phosphorus excretion of mixed zooplankton always overestimated the sum of excretion rates for healthy specimens, and considered that overestimation of the former was due to the release of nutrients by injured specimens. Ikeda *et al.* (1982) demonstrated that artificially injured copepods (*Acrocalanus gibber* and *Tortanus gracilis*) released phosphorus at a rate 6 to 9 times that of non-injured copepods (control). In the same set of experiments, artificial injury caused a twofold increase of ammonia excretion in *T. gracilis*, but no significant increase was seen in *A. gibber*. The death of a euphausiid, *Euphausia pacifica*, during incubation was observed to cause an eightfold increase in phosphorus excretion when compared to healthy specimens, but little increase in ammonia excretion was observed (Table 8.3 in Omori and Ikeda 1984).

Thus, it is clear that the effect of injured or dead specimens is most serious in determining phosphorous excretion rates, followed by ammonia excretion rates. The rapid release of phosphorus (PO_4^{-3}-P) from injured or dead specimens is considered to be due to hydrolysis of leached body fluids. While no relevant information is available at present, reduced oxygen consumption rates are expected in experiments where injured or

dead specimens are incubated together with healthy ones. In any case, if specimens die during an experiment, the data are best abandoned.

OXYGEN SATURATION

Solubility of oxygen decreases with increasing temperature and increasing salinity. Oceanic surface waters are nearly saturated. The oxygen concentration in sea water is expressed as ml per liter of sea water (1 ml $O_2\,l^{-1}$ = 1.43 mg $O_2\,l^{-1}$ = 44.6 μmol $O_2\,l^{-1}$ = 89.2 μg-at $O_2\ l^{-1}$), percent saturation against calculated saturation value from temperature and salinity, or barometric pressure (in mm of mercury, or Torr). The latter expression is commonly used in respiratory physiology and is 760 (gas pressure of 1 atmosphere) × 0.2095 (partial pressure of oxygen at STP) = 159 (mmHg, or Torr) for air-saturated sea water at 1 atmosphere.

In general, the oxygen consumption rate of marine animals is affected by the oxygen saturation of the surrounding sea water. For some animals (metabolic conformers) oxygen consumption rate is proportional to the concentration of oxygen, while for others (metabolic regulators) a constant oxygen consumption rate is observed as the oxygen concentration is reduced down to some critical level (Pc), below which the rate declines rapidly (Prosser 1961). However, intermediate types are also seen.

Marshall *et al.* (1935) observed a rapid decrease in the oxygen consumption rate of *Calanus finmarchicus* when the oxygen concentration decreased to below 3 ml $O_2\ l^{-1}$ (about 50% saturation). On the other hand, oxygen consumption rates of two mysid species (*Archaeomysis grebnitzkii* and *Neomysis awatschensis*) were not affected until the oxygen concentration decreased to 30% saturation, below which the rates decreased (Jawed 1973). An extreme capacity for respiring at low oxygen concentrations is reported for some midwater crustaceans (e.g. *Gnathophausia ingens*) from the oxygen minimum layer of the eastern Pacific (Teal and Carey 1967; Childress 1971, 1975). Ikeda (1977a) studied the relationship between oxygen consumption rate and oxygen saturation level for seven zooplankton species from Saanich Inlet, British Columbia. He observed no distinct Pc concentration for the seven species, but the effect of lowered oxygen concentration was less pronounced in *Calanus plumchrus* and *Holmesiella anomala*, collected from oxygen-deficient bottom water, than in species from oxygen-rich surface water. In comparing his oxygen consumption measurements on *Euphausia pacifica* with those of earlier workers, Paranjape (1967) found that his results were very low, although he used oxygen-saturated water, and concluded that they reflected the low oxygen concentration in the natural habitat of the *E. pacifica* used in his experiments.

For a realistic estimate of the oxygen consumption rate of zooplankton in the field, it is important to take into account not only the oxygen saturation in the experimental containers but also that in the field where the specimens were collected. In most oxygen consumption measurements with zooplankton from oxygen saturated environments, a reduction of the oxygen concentration to no less than approximately 80% saturation by the end of the incubation will safely avoid the effect of low oxygen.

TEMPERATURE

The relationship between various biological rate processes and temperature is frequently described by the Arrehenius relationship and the van't Hoff rule. The Arrehenius relationship describes the relationship between reaction rate (k) and absolute temperature (T);

$$k = A\exp(-E_a/RT) \tag{10.3}$$

where A is constant, E_a is the Arrehenius activation energy, and R is the gas constant (1987 kcal mol^{-1}). A plot of $\ln k$ against T^{-1} is therefore linear, with a slope of $-E_a/R$. The Arrehenius plot is a standard way of estimating activation free energies in enzyme kinetics, assuming strictly that the reaction involves only a single rate-limiting step (cf. Clarke 1987). For enzymes involved in the electron-transfer-system (ETS, see ETS Activity, section 10.4.1), E_a has been estimated as 16 kcal mol^{-1} for epipelagic zooplankton and 13.2 kcal mol^{-1} for bathypelagic zooplankton (Packard *et al.* 1975). The van't Hoff rule, which is described by the Q_{10} approximation, is the most commonly used way to describe the relationship between metabolic rates and temperature in marine zooplankton;

$$Q_{10} = (k_1/k_2)^{10/(t_1-t_2)} \tag{10.4}$$

where k_1 and k_2 are the rates corresponding to temperatures t_1 and t_2 (Prosser 1961). Q_{10} may be solved graphically, plotting the logarithm of k against t. Q_{10} is 2 to 3 for most biological rate processes (Prosser 1961).

The response of rate processes to temperature is defined according to the time scale involved as 'acclimation', 'acclimatization' and 'adaptation' (Clarke 1987). 'Acclimation' is the adjustment of rate processes to a new temperature in the laboratory, and 'acclimatization' is the adjustment of the rate processes to changes in environmental temperature (diurnal, seasonal). It should be noted that in laboratory acclimation it is usual to modify only temperature, keeping all other conditions constant. In contrast, acclimatization involves adjustment to the whole range of environmental variables characteristic of the field situation. Adaptation is an evolutionary adjustment, or genetic change accomplished at the population level to a daily or seasonal variation in temperature requiring acclimatization.

From an ecological viewpoint, there is a great deal of interest in the question of whether it is possible to predict the acclimatized or adapted metabolism of animals from laboratory acclimation experiments. Halcrow (1963) measured the oxygen consumption rate of *Calanus finmarchicus* acclimated to various temperatures and found that the rates were highly variable depending on the previous thermal history, time for acclimation, and season of collection. For specimens transferred acutely to higher temperature, 'overshoot' of the rate was observed before reaching the rate at a new stable level. Seasonal fluctuations of the oxygen consumption rate–temperature curves (so called R–T curves) have also been demonstrated in several neritic copepods acclimated in the laboratory (Anraku 1964; Gaudy 1973). Thus, accurate extrapolation of oxygen consumption data (and nutrient excretion data) of zooplankton obtained at field temperatures to rates at other temperatures in different seasons and locations is difficult using acclimation experiments. Clearly, maintenance of zooplankton at dissimilar temperatures between capture and experiment should be avoided to obtain valid metabolic rates of field zooplankton. Kinne (1964) reviewed diverse patterns of metabolic response in animals subjected to rapid temperature changes.

LIGHT

A large body of data indicates that full sunlight can be lethal to zooplankton and many other marine organisms (cf. review by Segal 1970) so that extreme care should be exercised when collecting zooplankton during daylight hours. Collection of zooplankton at night is the method of choice for use in metabolic experiments whenever feasible.

While most determinations of metabolic rate have been made in the dark or under subdued light to avoid excess activity of animals, illumination may affect the oxygen

consumption rate during daylight hours. Marshall *et al.* (1935) suspended glass bottles with *Calanus finmarchicus* at various depths in the sea and found an appreciable (100% or more) increase in its oxygen consumption rates when suspended above 5 m depth. For the specimens suspended below 5 m, no effect of sunlight was detected. Conover (1956) reported an increase in oxygen consumption rates of *Acartia tonsa* exposed to a single 20W fluorescent lamp in the laboratory, but he failed to find any light effect on *A. clausi* under the same conditions. *Euphausia pacifica* placed under light ($1.2-1.6 \times 10^2$ μW cm^{-2}) and dark ($< 1.8 \times 10^{-5}$ μW cm^{-2}) did not show any difference in oxygen consumption rate (Pearcy *et al.* 1969), but *Euphausia superba* consumed 30% more oxygen under the light (650 lx) than in complete darkness (Kils 1979). Fernández (1977) conducted extensive experiments on the rates of oxygen consumption, ammonia excretion and phosphorus excretion of several copepods under various intensities of sunlight (eight graded levels between 100% and 0%). According to his results, all rates increased in proportion to the amount of light above a certain threshold, which varied in different species, depending on the illumination at the depth where they were captured.

These results suggest that metabolic rates measured in the dark may underestimate rates during daylight hours for some epipelagic zooplankton. Whether or not to take into account light conditions depends on the vertical distribution pattern of zooplankton species of interest in the field. From a technical viewpoint, a drawback inherent with experiments in natural light conditions is the difficulty of reproducing day-to-day changes in weather. To best estimate metabolic rates in light sensitive zooplankton, laboratory experiments establishing the relationship between metabolic rate and light intensity, together with a field survey on natural light regimes of zooplankton, would be a good first step.

Evaluation of the effect of sunlight on zooplankton metabolism is becoming more important than before because of the reduction in the measurable ozone layer in the past several years and the resulting increase in incident solar mid-ultraviolet (UV-B, wave length 320–400 nm) radiation which damages DNA. The potential for deleterious effects on organisms living at the surface of both land and ocean is profound. To date, experimental study on this topic in marine zooplankton has been limited to the influence of UV-B dose/dose-rate on survival, fecundity and egg hatching success in several neritic copepods (Damkaer *et al.* 1980; Karansas *et al.* 1981; Dey *et al.* 1988). Surprisingly, the eyes of deep-sea pelagic crustaceans have been demonstrated to be sensitive to very low intensities of near-UV light (Frank and Widder 1994a, 1994b).

SALINITY

In offshore water, salinity is nearly constant in most locations. Therefore, in experiments on offshore zooplankton it is not necessary to consider salinity effects. However, for metabolic studies on zooplankton inhabiting near shore waters where spatial and temporal changes in salinity prevail, salinity may be one of the variables affecting the results.

Oxygen consumption rates of the copepods *Calanus finmarchicus* (Marshall *et al.* 1935) and *Centropages hamatus* (Anraku 1964) were depressed when individuals were placed in lowered-salinity water. In contrast, oxygen consumption rate of a typical estuarine copepod, *Acartia tonsa*, was found to increase in diluted sea water (101% increase at 30% sea water, Lance 1965). Dissimilar metabolic responses to lowered-salinity sea water may be explained by species-specific differences in tolerance to the reduced salinity. *Calanus finmarchicus* and *Centropages hamatus* are stenohaline species

and the decrease in oxygen consumption rates may have been due to physiological damage caused by low salinities below their tolerance limits. On the other hand, *A. tonsa* is a euryhaline species and its increased oxygen consumption rate may reflect higher energy requirements for osmotic and ionic regulation at low salinities. Increased oxygen consumption and ammonia excretion rates in diluted sea water have been reported on the Arctic under-ice amphipod *Onisimus glacialis* (Aarset and Aunaas 1990). In *O. glacialis*, increased ammonia excretion was interpreted as being due to increased use of NH_4^+ as a counter ion for Na^+ exchange during hyposmotic stress. The effect of salinity on nitrogen metabolism in crustaceans varies depending on the osmoregulatory capabilities of species. Nonetheless, it would be fairly safe to say that ammonia excretion rate increases when animals are hyper-regulating, and conversely, decreases when they are hypo-regulating (Regnault 1987).

FEEDING

The increased oxygen consumption rate in association with feeding is commonly called 'Specific Dynamic Action' (SDA) (Prosser 1961). Kiørboe *et al.* (1985) made detailed calculations of the energy budget of a copepod, *Acartia tonsa*, and concluded that the increased metabolism associated with SDA was largely related to biosynthesis and transport. Energy cost of feeding, gut activity, amino-acid oxidation and urea excretion were minor contributors to the total SDA. In other words, the SDA represents the 'cost of growth' rather than the 'cost of feeding' for the copepod. To better extrapolate metabolic data of zooplankton obtained in the laboratory to the field population, SDA and associated nutrient excretion need to be taken into account.

Technically, GF/C or GF/F filtered sea water is commonly used for the determination of zooplankton metabolic rates to avoid production or consumption of oxygen (or nutrients) by microorganisms in the sea water used for incubations. Since little is known of the natural foods and nutritional history of zooplankton at the time of capture, use of filtered sea water provides a common basis for interspecific comparison of various zooplankton from the sea. However, metabolic rates measured in non-feeding zooplankton may underestimate rates in the field.

Oxygen consumption rates of three copepod species incubated with algae (food) increased in proportion to their ingestion rates (Gaudy 1974). A positive correlation between oxygen consumption rate and ingestion rate has also been reported on *Euphausia superba* (Ikeda and Dixon 1984). Conover and Lalli (1974) measured the rate of *Clione limacina* in the presence of the prey (*Spiratella retroversa*) and found the rate to increase 2.0 to 3.5 times during feeding. Vidal (1980), however, failed to find any significant effect of the presence of food on the oxygen consumption rate of *Calanus pacificus*.

To determine nutrient excretion rates of zooplankton feeding on phytoplankton, a correction for the simultaneous uptake of zooplankton excreta by phytoplankton must be made. The magnitude of this correction will vary depending on the concentration of phytoplankton and excreta. Figure 10.2 shows the results of excretion experiments in which *Metridia pacifica* was incubated in sea water containing various amounts of phytoplankton (*Skeletonema costatum*). Apparent excretion rates of ammonia and phosphorus tended to increase with increasing phytoplankton concentration, but both rates dropped rapidly at the maximum phytoplankton concentration. Phosphorous uptake by phytoplankton was so great that the apparent excretion rate of phosphorus became negative. To estimate the real excretion rate of *M. pacifica* during feeding, Takahashi and Ikeda (1975) corrected for phytoplankton uptake of ammonia and

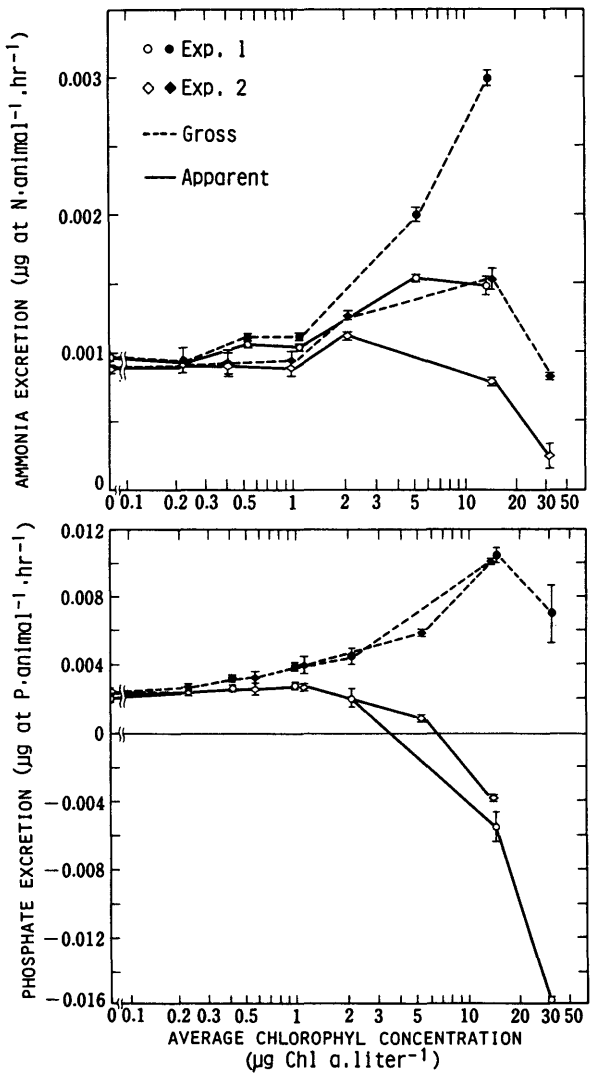

Fig. 10.2 Apparent and gross rates of ammonia and inorganic phosphorus excretion by *Metridia pacifica* incubated in bottles (900 ml) with different concentrations of phytoplankton (*Skeletonema costatum*) for 24 h at 8 °C. About 100 specimens of *M. pacifica* were used in each bottle. Concentration of phytoplankton is expressed as chlorophyl *a*. Gross excretion rates are calculated by correcting for the uptake by phytoplankton of ammonia and inorganic phosphorus during incubation (After Takahashi and Ikeda 1975, with permission).

phosphorus by applying Michaelis–Menten uptake kinetics for the phytoplankton they used. Excretion rates thus corrected are also shown in Figure 10.2 as gross excretion rates. The increase in the gross excretion rate in relation to phytoplankton concentration reflects a proportional increase of zooplankton feeding on phytoplankton. In a similar study, Lehman (1980) estimated ammonia and phosphorus excretion rates of *Daphnia pulex* feeding on *Chlamydomonas reinhardtii*. To correct for nutrient uptake by

Chlamydomonas, he added both nutrients in excess to the incubation medium of *Daphnia* so as to achieve a constant nutrient uptake by *Chlamydomonas*. Michaelis–Menten kinetics saturate at high nutrient concentrations, thus nutrient uptake by *Chlamydomonas* becomes independent of nutrient concentration. Adopting Lehman's (1980) procedure, Miller and Landry (1984) determined the ammonia excretion rate of *Calanus pacificus* feeding on *Thalassiosira weissflogii*, and concluded that the excretion rate of *C. pacificus* was independent of ingestion rate.

Caperon *et al.* (1979) measured ammonia excretion of microzooplankton in natural sea water using a ^{15}N isotope dilution technique. In this method, ammonia labeled with ^{15}N is added to natural sea water. The decrease in ^{15}N ammonia as a fraction of the total ammonia ($^{15}N + {}^{14}N$) is due to its dilution by microzooplankton excretion. This method is an alternative approach to the analysis of zooplankton–phytoplankton interactions in zooplankton excretion studies.

In an attempt to simplify the experimental design for assessing the effects of feeding on nutrient excretion by *Daphnia pulex*, Vanderploeg *et al.* (1986) used heat-killed algae which does not take up or release nutrients. However, application of this procedure requires caution, as Conover *et al.* (1988) found that heat-killed algae were less acceptable as food for the copepod *Pseudocalanus* sp., resulting in depressed rates of oxygen consumption and ammonia excretion.

Clearly, metabolic rates in zooplankton show considerable variability with feeding. Results obtained using various methods show little consistency. Considering the complex interactions between prey and predators it is conceivable that these dissimilar results may partly be due to differential experimental methods, different kinds of prey organisms, and species-specific differences in nutritional and metabolic status of predators.

DIEL RHYTHM

Biological rhythms are composites of an endogenous physiological pacemaker which generates the basic frequency, coupled to an environmental cycle which adjusts the phase of the internal clock to local time (DeCoursey 1983). 'Circadian' is the term for the endogenous rhythm repeating about every 24 h (from *circa dia* meaning 'about once a day'). From technical viewpoints, a diel rhythm in zooplankton metabolism, if it existed, would be a possible source of error in estimating daily metabolism from short-term experiments. Diel metabolic rhythmicity is a complex phenomenon that may couple feeding, swimming behavior and spawning to diel cycles of light, temperature, oxygen concentration and other environmental variables. Biological rhythms are often difficult to distinguish from purely exogenously controlled behaviors without experiments in which environmental factors are strictly controlled (cf. DeCoursey 1983).

Endogenous biological rhythms are characterized by a great 'inertia' and therefore may persist for quite a long time after an organism has been isolated from its natural environment (Mezykowski and Rakusa-Suszczewski 1979; DeCoursy 1983). On this premise, Pearcy *et al.* (1969) placed individual *Euphausia pacifica*, a species which is known as an oceanic diel vertical migrator, in Warburg flasks and monitored its oxygen consumption rate over 32 h in continuous darkness. The results showed no diel rhythm in the oxygen consumption rates of this euphausiid; this finding is further supported by the absence of a light–dark effect on the rate of this species. However, similar experiments on mixed freshwater zooplankton and *Diaptomus kenai* by Duval and Geen (1976) detected a common diel rhythm in the oxygen consumption rates in each; the rates were bimodal, with maximum values at dawn and dusk. Pavlova (1994) determined oxygen consumption rates at different times of the day on copepods that

normally perform a diel vertical migration (*Pleuromamma xiphias, Calanus euxinus*), and found significantly higher rates in those specimens collected at night. In the same study, Pavlova (1994) noted that no day–night differences were seen in copepods in which diel vertical migration was less evident (*Acartia clausi, Temora discaudata*). Checkley *et al.* (1992) investigated diel rhythmicity in ammonia excretion rates of the migratory copepods *Acartia* sp. and *Centropages furcatus*. Specimens collected at various times within a day were used in incubation experiments (this was in contrast to the use of a stock of specimens as in the experiments of Pearcy *et al.* 1969; Duval and Geen 1976), and found that the daytime rates were 2.5 to 5 times greater than the night-time rates (Figure 10.3).

Evaluation of endogenous diel rhythmicity in metabolic rates may not be necessary for the studies in which the major emphasis is daily metabolism (metabolic rate integrated over one day). In theory, if starvation is not a problem, this can be achieved by

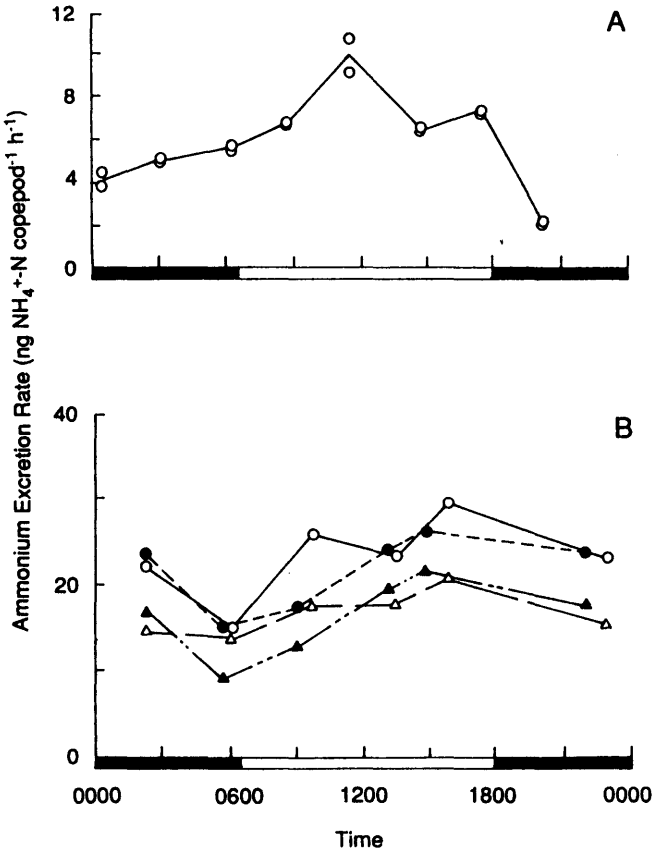

Fig. 10.3 Ammonia excretion rate of adult female copepods collected at ca. 3 h intervals for 24 h, and incubated in filtered sea water for 3–4 h under simulated *in situ* conditions of light and temperature. (A) *Acartia* sp. at 25 °C. Symbols represent duplicate ammonia analyses. (B) *Centropages furcatus* at 22–30 °C. Symbols: open circle, specimens from shallow warm; closed circle, those from deep warm; open triangle, those from shallow cold; closed triangle, those from deep cold waters (after Checkley *et al.* 1992, with permission).

incubating zooplankton for time periods of one half day or one full day. The diel cycles of some important environmental variables will then be accomplished within the laboratory experiments. The following is an example in which only cyclic changes in ambient temperature are taken into account in the estimation of daily metabolism.

The hyperiid amphipod *Themisto japonica* is a typical oceanic diel vertical migrator. During the course of its migration from the surface to 350 m depth everyday, the amphipod encounters a change in temperature from 15 °C to 1 °C in summer. Using a programmable water bath, Ikeda (1992) simulated the daily fluctuation of temperature for migrating *T. japonica*, and compared oxygen consumption rates of specimens incubated in this fluctuating temperature bath for 1 day (= 1 cycle of temperature change) with rates of those maintained in a constant temperature bath. The constant temperature bath was set to 8 °C (integrated daily mean temperature). The daily oxygen consumption of *T. japonica* placed in the fluctuating temperature bath did not differ significantly from that of *T. japonica* in the constant temperature bath. While the associated change in hydrostatic pressure encountered by *T. japonica* was ignored in the experiment, previous findings (e.g. Torres and Childress 1983) suggested that effects of pressure on metabolism over the depth range of *T. japonica* would be minimal (see page 472). Thus, daily metabolism of migrating *T. japonica* can be estimated from the constant temperature experiment in which the temperature was carefully adjusted to its daily mean. Similar results were obtained for *Euphausia pacifica* by Torres and Childress (1983). Apparently, life in fluctuating temperature serves mainly to eliminate the short term metabolic overshoots and undershoots typically seen in the metabolism of acclimated individuals when temperature is acutely raised or lowered (cf. Prosser 1961).

BACTERIA

Bacterial growth during incubation is unavoidable and may be a source of error when measuring rates of oxygen consumption and/or nutrient excretion using the sealed chamber method. Marshall and Orr (1958) used antibiotics, such as streptomycin and chloromycetin (50 mg l^{-1} of each), to minimize bacterial oxygen consumption during their experiments on oxygen consumption and feeding in *Calanus finmarchicus*. Presence or absence of antibiotics did not affect the oxygen consumption rate of the copepod, but some copepods ceased feeding after addition of antibiotics. No appreciable effect of antibiotics (streptomycin, 50 mg l^{-1}) was seen in the oxygen consumption rates of *Neocalanus plumchrus* (Ikeda 1970).

For nutrient excretion experiments, Hargrave and Geen (1968) incubated several zooplankton species with and without antibiotics (penicillin and streptomycin), to measure excretion of phosphorus (inorganic and organic). In all species the excretion rates were lower for the specimens incubated without antibiotics, thus showing bacterial uptake of phosphorus during incubation. Mayzaud (1973) observed similar results for organic nitrogen excretion by *Meganyctiphanes norvegica*, but not in *Acartia clausi* in the same experiment. Jawed (1969) maintained *Neomysis rayii* and *Euphausia pacifica* with antibiotics prior to nitrogen (ammonia, amino acids, urea and total nitrogen) excretion measurements in autoclaved sea water and bottles. The excretion rates of all forms of these treated specimens were not significantly different from the rates of untreated specimens.

Bacteria may be either a net regenerator or consumer of nutrients, depending on their growth rate and physiological state (Goldman *et al.* 1987). In this light, not only the differences in zooplankton species and in experimental designs, but also the physiological condition of bacteria involved in each experiment may contribute to inconsistent

results between studies. As in the oxygen consumption experiments above, the use of antibiotics requires caution concerning appropriate dosage. Some antibiotics interfere with the ultraviolet method for nitrogen analysis (Butler *et al.* 1969). Overall, the degree to which bacteria affect the measurement of nutrient excretion by marine zooplankton is presently unclear.

MOLTING

Molting, when it occurs during the incubation of individual crustacean zooplankton, may result in overestimates of routine metabolism. In euphausiids, molting is known to accelerate oxygen consumption rates (Paranjape 1967; Ikeda and Mitchell 1982). Bulnheim (1972) made detailed measurements of increased oxygen consumption (2.2–3.9 times the rate at non-molting) during the course of molting in benthic gammarid amphipods. Not only oxygen consumption, but also phosphorus excretion rate in euphausiids (Ikeda and Mitchell 1982), and ammonia excretion rate in decapod crustaceans (Regnault 1987) has been observed to increase when specimens molt during incubation.

HYDROSTATIC PRESSURE

Hydrostatic pressure increases progressively with increasing depth (1 atmosphere or 101.3 kPa per 10 m depth). Early interest in the effect of hydrostatic pressure on zooplankton metabolism mainly concerned diel vertical migrators, particularly residents of the deep scattering layer that experience large pressure changes ($\geq$20 atm) twice daily as a result of their vertical excursions. Oxygen consumption rates of diel vertical migrators such as euphausiids (Teal and Carey 1967; Pearcy and Small 1968), decapods (Teal 1971), and thecosomatous pteropods (Smith and Teal 1973) determined under various combinations of temperature and hydrostatic pressures in the laboratory yielded a consistent result that the rates were affected by temperature but not by pressure. The lack of appreciable hydrostatic pressure effects on oxygen consumption rates has recently been confirmed on bathypelagic chaetognaths, hydromedusae and a polychaete by comparing rates at 1 atm and at 100 atm in the laboratory (Childress and Thuesen 1993). All these results suggest that estimation of oxygen consumption rates of zooplankton living at depth is experimentally feasible at 1 atm, provided that live-specimens are recovered successfully to the surface without damage. In this regard, Bailey *et al.* (1994) noted a loss in motor activity of delicate mesopelagic gelatinous zooplankton due to decompression, which was reflected in reduced oxygen consumption rates.

It is well documented that the metabolism of pelagic crustaceans, fishes and cephalopods declines with the increase of depth of occurrence (Childress 1975; Quetin *et al.* 1980; Ikeda 1988; Torres *et al.* 1979, 1994; Childress 1995). This depth-related decline of crustacean and fish metabolism is not due to the increase of hydrostatic pressure as mentioned above, nor to the decrease of temperature; it is an adapted characteristic of many species of deeper-living fauna (Childress *et al.* 1980; Childress and Mickel 1985; Ikeda 1988). A comparison of oxygen consumption rates and intermediary metabolic enzyme activities in chaetognaths living at various depths by Thuesen and Childress (1993a, 1994), revealed that a depth-related decline in metabolism was not the case for chaetognaths and medusae. Thuesen and Childress (1993a, 1994) and Childress (1995) postulate that a depth-related change of metabolism would be selected for in visual predators that require a well-developed musculature to chase down prey that can be visually targeted in well-lit surface waters, but not in visual predators in the light-

limited deep-sea. The limited distances for visual detection afforded by the low light levels at depths inhabited by deeper living species has resulted in a loss of locomotory musculature and a concomitant decline in metabolic rates. The same argument does not apply to non-visual predators such as chaetognaths whose methods of prey detection are not appreciably altered with depth. In light of the diversity of pelagic fauna in the deep-sea, more data are needed to prove or disprove the present hypothesis for reduced metabolic rates of deep-living zooplankton.

TURBULENCE

In the sealed chamber method for measuring metabolic rate, zooplankton are typically confined in standing (turbulence-free) water during the experiment. In nature this is not the case, and zooplankton living in the shallow layers of the sea are more or less under the influence of small-scale turbulence at all times. Small-scale turbulence can affect zooplankton metabolism in two ways: (1) by increasing the encounter probability between zooplankton and food particles, and (2) by increasing the frequency of escape reaction of zooplankton, a metabolically expensive swimming behavior (Saiz and Alcaraz 1992).

Saiz and Alcaraz (1992) placed *Acartia* sp. in 25- to 50-ml screw-cap polyethylene vials three-quarters filled with GF/F filtered, air-saturated sea water, and the vials were placed on a reciprocal shaker (90–100 strokes min^{-1}, 2.5 cm amplitude) for 24 h to determine their ammonia and phosphorus excretion rates. As a control, *Acartia* sp. was placed in standing vials. The excretion rates of *Acartia* sp. placed on the shaker were 1.6 times higher on average than the rates of controls. Saiz and Alcaraz (1992) noted that the intensity of turbulence generated by shaking in their experiments was not quantified, but was probably higher than that found in the field, at least in oceanic waters. Clearly, oxygen consumption is superior to nutrient excretion for evaluating the effects of small-scale turbulence on zooplankton metabolism. In a later study, Alcaraz *et al.* (1994) determined heartbeat rates, instead of oxygen consumption rates, of several zooplankton species (both marine and freshwater) under quantified turbulence (ca. 5 $mm^2\ s^{-3}$) and observed increases ranging from 5% to 93%. However, a poor correlation between heartbeat and oxygen consumption rates was reported for *Euphausia superba* and *Parathemisto gaudichaudi* by Opalinski (1979). More investigation is needed to evaluate and generalize the effect of small-scale turbulence on zooplankton metabolism. Clearly, development of new experimental techniques and quantitative evaluation of turbulence levels encountered by zooplankton in the field will help to resolve the interactions between turbulence and metabolism in zooplankton.

10.3.2 Body size and temperature as bases of metabolic comparison

Metabolic rate (M) of animals is known to vary as a function of body mass (W) intraspecifically and interspecifically (Zeuthen 1947, 1953; Hemmingsen 1960). The relationship is expressed as

$$M = aW^b, \tag{10.5}$$

where a and b are constants. According to Zeuthen's (1953) recapitulation theory the mass exponent b varies successively with the increase of body mass, from 0.75 to 1.0, then to 0.75, where the middle 1.0 is typical in very small metazoans phylogenetically or mid-development stages ontogenetically. However, Banse (1982) re-analyzed published data of metabolism–body mass relationships of very small invertebrates, concluding that the b

for this group of animals was near 0.75 instead of 1.0. Thus, Zeuthen's theory appears not to be warranted any more (Banse 1982). For marine planktonic metazoans of which body mass ranges five to six orders of magnitude at most, the mass exponent b is characterized as less than 1 and usually in the range of 0.7 to 0.9. Within narrow body mass ranges the relationship between metabolic rate and body mass is often masked by the scatter of the data, i.e. the confidence interval of b is too wide to judge $b < 1$. From a statistical viewpoint, a geometric (GM) regression model, rather than arithmetric (AM) regression model (= the least-square regression), is appropriate for calculating b (Ricker 1973; Laws and Archie 1981). A body mass exponent calculated from AM regression (b) and that (d, i.e. $M = cW^d$, where c and d are newly designated constants) from GM regression are the same when the correlation coefficient (r) is 1.00, but the former is greater than the latter when $r < 1.00$, and $r < 1.00$ is usually the case (i.e. $d = b/r$ cf. Ricker 1973).

For metabolic comparison between species with dissimilar body masses, a common constant b needs to be established or assumed (or else the conclusion will vary depending on the choice of body mass). The results of metabolic comparison are also affected by the expression of body mass when the body composition of the species to be compared is different. Ikeda and Mitchell (1982) compared oxygen consumption rates of *Euphausia superba* and *Salpa thompsoni* on the basis of equivalent body mass and noted that the rate of the former was greater than the latter by a factor of 17 on a wet weight, 4 on a dry weight, and 0.8 on a carbon or nitrogen basis. Carbon or nitrogen units appear to be superior to wet and dry weight to reduce the phylogenetic differences in body composition for the purpose of interspecific metabolic comparison. Schneider (1990) reached the same conclusion from the body carbon based comparison of ammonia excretion rates between gelatinous and nongelatinous zooplankton. Ikeda (1988) proposed 'Adjusted Metabolic Rate' (AMR) for metabolic comparison between mesopelagic and epipelagic zooplankton. AMR is defined as M divided by (body nitrogen)$^{-b}$. The mass exponent b used in the comparison of Ikeda (1988) was 0.8505 for oxygen consumption rate, 0.8361 for ammonia excretion rate, and 0.8704 for phosphorus excretion rate, all of which are derived from statistical analysis of comprehensive data sets describing epipelagic zooplankton metabolism collected from several regions of the world ocean (cf. Table 10.2).

The relationship between metabolic rates (M) and temperature (T°C) is frequently described by Q_{10} (see Temperature, section 10.3.1). When the Q_{10} is constant over a given temperature range, the relationship is re-expressed as $M = \alpha\beta^T$, where α and β are constants. Since it is known already that temperature effects are difficult to analyze using laboratory acclimation experiments (see Temperature, section 10.3.1), a comparison of metabolic rates of zooplankton living in dissimilar thermal regimes is most appropriate for drawing generalized conclusions regarding temperature effects. Intraspecific comparisons would be ideal, but the data presently available are not sufficient for this analysis. Interspecific comparison is the only alternative.

Ikeda (1985) compiled oxygen consumption data of 143 zooplankton species, ammonia excretion data on 131 species, and phosphorus excretion data on 52 species (general size range: 10^{-3} to 10^3 mg dry weight) from tropical, subtropical, temperate, subarctic and Antarctic waters (temperature range: -1.4 to 30 °C) and analyzed the data as a function of body mass and habitat temperature, assuming a constant mass exponent b in metabolism–body mass relationship and a constant Q_{10} over the temperature ranges investigated. His results revealed that the 94% to 95% of the variation in oxygen consumption rates could be ascribed to habitat temperatures and body sizes of zooplankters (Table 10.2). Excretion rates of ammonia and inorganic phosphorus were

Table 10.2 Regression statistics of metabolic rates (Y = oxygen consumption, ammonia excretion, or inorganic phosphorus excretion rates) on body mass (X_1 = dry (DW), carbon (CW), nitrogen (NW), or phosphorus (PW) weight) and habitat temperature (X_2) for marine zooplankton. Q_{10} calculated from a_2 are shown on the right-hand side of the table ($Q_{10} = \exp(10 \times a_2)$) (modified from Ikeda 1985).

			$\ln Y = a_0 + a_1 \ln X_1 + a_2 X_2$				
Metabolic rate	**Body mass unit**	**N**	a_0	a_1	a_2	R^2	Q_{10}
Oxygen uptake	DW	721	−0.2512	0.7886	0.0490	93.9	1.632
	CW	721	0.5254	0.8354	0.0601	95.5	1.824
	NW	721	1.7412	0.8505	0.0636	95.1	1.889
	PW	721	3.7890	0.8167	0.0552	94.0	1.737
Ammonia excretion	DW	1 186	−2.8900	0.7616	0.0511	85.4	1.667
	CW	1 186	−2.1763	0.8293	0.0648	86.5	1.912
	NW	1 186	−0.9657	0.8361	0.0656	86.2	1.927
	PW	1 186	1.0708	0.8063	0.0562	84.5	1.754
Phosphate excretion	DW	749	−4.3489	0.7983	0.0285	86.4	1.330
	CW	749	−3.6031	0.8622	0.0438	86.5	1.550
	NW	749	−2.3490	0.8704	0.0441	86.9	1.554
	PW	749	−0.1839	0.8569	0.0376	84.2	1.443

more variable; 84% to 87% of their variability was explained by changing temperature and body mass. Because of the wide variability in the characteristics of the studied zooplankton species, Q_{10} varied as a function of the body mass unit chosen for comparison. General ranges were 1.63 to 1.89 for oxygen consumption rate, 1.67 to 1.93 for ammonia excretion rate, and 1.33 to 1.55 for phosphorus excretion rate. The combination of body mass-specific Q_{10} with AMR can allow standardization of metabolic rates in terms of body mass and temperature as bases for metabolic comparison of zooplankton.

The data analyzed in Table 10.2 do not include metabolic data of Arctic zooplankton. Because of the interest in historical 'metabolic cold adaptation', oxygen consumption rates of Arctic species are of special interest, i.e. despite a similar sub-zero habitat temperature the rates of Arctic zooplankters are expected to be lower than the rates of Antarctic zooplankters because the Arctic ecosystem is younger. For a detailed historical account of this hypothesis see Holeton (1974) and Clarke (1983). An intra-generic comparison of oxygen consumption rates in selected zooplankton species from the Arctic and Antarctic revealed no significant difference between the two (Ikeda 1989a). Thus, the regression equation in Table 10.2 can be used as the basis for metabolic comparison of zooplankton living over world oceans.

As an obvious divergence from the regression equation in Table 10.2, extremely reduced metabolic rates have been reported for overwintering copepods in 'diapause'; i.e. *Calanus pacificus californicus* in the Santa Barbara Basin (Alldrege *et al.* 1984), *Calanus finmarchicus* and *C. helgolandicus* in Norwegian fjords (Hirche 1983), *Calanoides acutus* in the Weddel Sea (Drits *et al.* 1994), *Neocalanus cristatus, N. plumchrus* and *Pseudocalanus minutus* in the Japan Sea (Ikeda and Hirakawa 1998). These copepods are primarily herbivores living in cold water, and their diapause stages (mostly late copepodite stages) are characterized by their occurrence from the mesopelagic zone, motionless in behavior and a large accumulation of lipids (stored energy) in the body. For a recent account of dormancy (including diapause) of planktonic copepods, see the review of Williams-Howze (1997).

10.3.3 Metabolic quotients

Simultaneous measurements of oxygen consumption, ammonia excretion and inorganic phosphorus excretion allow the ratios of oxygen consumption to ammonia excretion (O:N), ammonia excretion to inorganic phosphorus excretion (N:P), and oxygen consumption to inorganic phosphorus excretion (O:P) to be compared on an atomic basis. These ratios, called metabolic quotients, change depending on the metabolic substrates of the animal. Nitrogen and phosphorus contents of animal protein, lipid, and carbohydrate, together with the amount of oxygen required for combustion of each class of biomolecule are summarized in Table 10.3. The O:N, N:P and O:P ratios for each class of organic matter are also calculated. It must be noted that these ratios will change slightly, because of diversity within a class of biomolecule, particularly in the make-up of protein and lipid, and no precise information is available for zooplankton material. Even so, consistent variations are observed in these ratios depending on whether the metabolic substrate is protein, carbohydrate or lipid. It is apparent from Table 10.3 that a carbohydrate-dominated metabolism causes high O:N and O:P ratios. Low N:P and O:N ratios are characteristic of lipid- and protein-oriented metabolisms respectively.

Raymont and Krishnaswamy (1960) and Raymont and Conover (1961) observed little

Table 10.3 Average nitrogen and phosphorus composition of organic matter, the oxygen required to oxidize each class of organic matter in an animal body, and calculated metabolic quotients (O:N, N:P and O:P, by atoms) (modified from Ikeda 1974).

	Carbohydrate	Lipid	Protein
N (g/g)[a]	0	0.0061	0.178
P (g/g)[a]	0	0.0213	0.007
O (l/g)[b]	0.83	2.01	1.02
O:N	∞	412	7.2
N:P	—	0.63	56
O:P	∞	261	403

[a] *From Rogers (1927)*
[b] *From Gnaiger (1983)*

change in the carbohydrate content of starved zooplankton. In conjunction with these observations, the carbohydrate content of zooplankton was found to be only a few percent of the dry weight (5% at most). Even if all the carbohydrate in a zooplankter was metabolized, it would not be large enough to support the animal's metabolic requirement for one day. Therefore, protein and lipid are considered the major metabolic substrates of starved zooplankton. According to Conover and Corner (1968), the O:N ratio of some boreal zooplankton is high at the end of summer but declines throughout winter, with a corresponding decrease in lipid. During the spring phytoplankton bloom, zooplankton feed actively on phytoplankton and store lipid, but the O:N ratio remains low during this period. The O:N ratio increases only after the zooplankton have deposited a large amount of lipid, indicating a close association of the O:N ratio to the lipid content of zooplankton.

Assuming that the respective nitrogen content of protein and lipid is 16% and 0%, and the oxygen required for complete combustion per 1 g for protein and lipid is 1.04 and 2.02 l, Ikeda (1974) calculated an O:N ratio of 24 when protein and lipid are metabolized in equal quantities at the same time; hence an O:N ratio less than 24 indicates protein-oriented metabolism and a ratio greater than 24 indicates lipid-oriented metabolism (from the data in Table 10.3 this is re-calculated to be 20.6). O:N ratios were used to compare the metabolic substrates of zooplankton inhabiting a large geographical area extending from tropical through subarctic waters. As a result, he concluded that zooplankton living in tropical, subtropical and temperate waters were characterized by protein metabolism, while those living in subarctic waters showed a wide range in the O:N ratio. In addition to season (Conover and Corner 1968) and specific differences (Ikeda 1974; Ikeda and Mitchell 1982; Gaudy and Boucher 1983; Quetin *et al.* 1980; Ikeda and Skjoldal 1989), O:N ratios are known to change as a result of starvation (Mayzaud 1976; Ikeda 1977b), recent feeding conditions of animals (Ikeda and Dixon 1984), and acclimated temperature (Mayzaud and Conover 1988). Extremely low O:N ratios (lower than the theoretical minimum of 7.2, see Table 10.3) seen in the results of some workers in Table 10.4 may be caused by artificially high excretion rates of animals, the result of severe stress either in the field or in the laboratory (Mayzaud and Conover 1988). Mayzaud and Conover (1988) reviewed the O:N ratio in marine zooplankton and noted that while the O:N ratio reflected changes in the biochemical composition of the body for zooplankton with large energy reserves (lipid), the ratio for

Table 10.4 The O:N, N:P and O:P atomic ratios from the measurements of oxygen consumption, ammonia excretion and phosphorus excretion rates being reported by previous workers.[a]

Zooplankton	O:N	N:P	O:P	References
Mixed zooplankton (UFW)	7.7	7.0	54.0	Harris (1959)
Mixed zooplankton (UFW)	41.0	9.98	222.0	Martin (1968)
Calanus cristatus (UFW)	5.7	19.0	110.0	Taguchi and Ishii (1972)
Calanus plumchrus (UFW)	6.8	13.0	89.0	Taguchi and Ishii (1972)
Mixed zooplankton (UFW)	13.48	10.33	142.4	Le Borgne (1973)
Sagitta hispida (FW)	—	11.3	—	Beers (1964)
Mixed zooplankton (UFW)	—	—	72.0	Satomi and Pomeroy (1965)
Calanus helgolandicus (FW)	9.8–15.6[b]	—	—	Corner *et al.* (1965)
Boreal zooplankton (10 species) (UFW)	6–200[b]	—	—	Conover and Corner (1968)
Calanus finmarchicus (FW)	—	10.8	—	Butler *et al.* (1969)
Calanus finmarchicus (FW)	—	11.0[c]	—	Butler *et al.* (1970)
Sagitta hispida (FW)	6.8	—	—	Reeve (1970)
Calanus helgolandicus (FW)	—	16.5[c]	—	Corner *et al.* (1972)
Temora stylifera (FW)	7–15[d]	—	—	Nival *et al.* (1974)
Mediterranean zooplankton (4 species) (FW)	1.6–12.1	—	—	Mayzaud (1973)
Boreal, temperate, subtropical and tropical zooplankton (81 species) (FW)	4–115	—	—	Ikeda (1974)
Mixed zooplankton (UFW)	—	6.8	—	Mullin *et al.* (1975)
Mesopelagic crustaceans (14 species) (FW)	9.1–91.0	—	—	Quetin *et al.* (1980)
Antarctic zooplankton (14 species) (FW)	7.0–19.8	2.5–24.7	43–304	Ikeda and Mitchell (1982)
Tropical zooplankton (27 species) (FW)	2.7–28.6	2.3–44.5	19.6–410	Gaudy and Boucher (1983)
Calanus glacialis (FW)	3.0–9.9	7.7–12.9	38.7–76.5	Båmstedt and Tande (1985)
Tropical ctenophores (4 species) (FW)	10.2–15.8	—	—	Kremer *et al.* (1986)
Salps (10 species) (UFW, FW)	13–28	—	—	Cetta *et al.* (1986)
Antarctic mesopelagic zooplankton (7 species) (FW)	14.1–73.9	1.8–15.9	67–290	Ikeda (1988)
Arctic zooplankton (10 species) (FW)	7.0–19.8	2.5–24.7	43–304	Ikeda and Skjoldal (1989)

[a] Use of unfiltered (UFW) and filtered (FW) sea water for measurement is noted. — = no data. [b] Ninhydrin N. [c] Total N/total P. [d] Taken from Nival et al. 1974, Figure 10.

small neritic zooplankton with few energy reserves reflected the quality and quantity of food in the environment.

The N:P and O:P ratios are not as sensitive as the O:N ratio for assessing metabolic substrates of zooplankton, though in earlier studies interest centered around the constancy of N:P and O:P values. Satomi and Pomeroy (1965) compared the O:P ratio of mixed zooplankton from three different sites and obtained quite similar values (63–75, with a mean of 72). From a seasonal study of total nitrogen and total phosphorus excretion by *Calanus finmarchicus*, Butler *et al.* (1970) found the atomic ratio of N:P was stable through spring (11.0) and winter (14.6). If the N:P and O:P ratios are constant, estimation of oxygen consumption, nitrogen excretion and phosphorus excretion rates can be made by measuring only one of these variables. However, subsequent studies by other workers revealed that there is a large variation in these ratios (Table 10.4). The N:P and O:P ratios are now known to change not only between species (Ikeda and Mitchell 1982; Gaudy and Boucher 1983; Ikeda and Skjoldal 1989) but also within the same species, depending on temperature (Le Borne 1982), time for incubation (Le Borne 1979; Ikeda and Skjoldal 1980; Båmstedt and Tande 1985), quality and quantity of food given (Ikeda and Dixon 1984), and prolonged starvation of animals over weeks (Ikeda 1977b). Since an abnormally high phosphorus release has been observed in damaged zooplankton (see Injury/death in section 10.3.1), extremely low N:P and O:P ratios may be used as indicators of damage in experimental animals.

Redfield *et al.* (1963) proposed the average C:N:P composition of marine phytoplankton and zooplankton as 106:16:1 by atoms. Richards (1965) constructed a model of the organic matter composition of phytoplankton and zooplankton based on Redfield's ratio and a sequence of decomposition:

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 106O_2 = 106CO_2 + 16NH_3 + H_3PO_4 + 106H_2O \quad (10.6)$$

$$16NH_3 + 32O_2 = 16HNO_3 + 16H_2O. \quad (10.7)$$

Hence,

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 138O_2 = 106CO_2 + 122H_2O + 16HNO_3 + H_3PO_4. \quad (10.8)$$

From this scheme, the O:N, N:P, and O:P ratios are predicted to be 17, 16, and 276, respectively. When biological oxidation by zooplankton is considered, the nitrogenous end product is not NHO_3 but NH_3. Thus, the appropriate ratios would be 13, 16, and 212. Application of these ratios to individual zooplankton species is cautioned, since a large departure of the C:N:P ratio from that of Redfield has been noted (cf. Corner and Davies 1971).

10.4 METABOLIC RATE AND ENZYMATIC INDICES

The search for physiological indices to characterize field-caught specimens has been slowly gaining momentum in biological oceanography for the last 30 years. Physiological indices may be thought of as biochemical proxies for a physiological rate that is either difficult to measure or where time constraints make sufficient data collection difficult in the time frame of a single cruise. The development of reliable enzymatic indices will help resolve the large hysteresis between the data gathering efforts of physical and chemical

oceanographers and zooplankton biologists. In a modern oceanographic survey the CTD-rosette and automated nutrient analyzers allow physicists and chemists to complete much of their data-gathering and often some analysis before leaving the ship. Similarly, phytoplankton biologists are now able to map chlorophyl biomass in real-time. In contrast, zooplankton biologists often leave the ship with a wagon-load of samples that take months to analyze and little if any information on the physiological well-being of the zooplankton they have collected, even in a relative sense throughout the survey area. New methods such as optical plankton counters (see Chapter 7) may help to resolve the time-lag in analysis of distribution and abundance of zooplankton. Likewise, enzymatic methods may help to describe physiological status of zooplankton more quickly than traditional incubation methods, and further, will work on frozen specimens. As is the case with optical plankton counters, enzymatic methods will also result in some loss of resolution. It will become obvious as the field develops whether the loss in resolution is acceptable.

Biochemical indices are best grouped by the physiological rate that they are acting as a proxy for. Four physiological rates have been addressed by investigators using biochemical proxies: ingestion rate, growth rate, oxygen consumption rate, and excretion rate. Ingestion and growth rates are covered elsewhere (Chapters 8 and 9, respectively) leaving us to consider proxies for respiration and excretion in this chapter. Biochemical indicators of respiration in zooplankton include succinate dehydrogenase, first described by Curl and Sandberg (1961), electron transfer system (ETS) activity, developed by Packard (1969), lactate dehydrogenase (LDH) and pyruvate kinase (PK) activity (Berges *et al.* 1990, Berges and Ballantyne 1991), citrate synthase (CS) activity (Berges *et al.* 1990, 1991; Clarke *et al.* 1992; Clarke and Walsh 1993) and, malate dehydrogenase (MDH) activity (Thuesen and Childress 1993a, 1993b, 1994). By far, ETS activity is the most widely employed index of respiration in oceanography today, although the enzymes of intermediary metabolism (LDH, CS, PK, MDH) show considerable promise as physiological indices as well. Ammonia excretion can be estimated using the activity of glutamate dehydrogenase (GDH, Bidigare and King 1981) a key enzyme in the transamination reactions of the cell. Mayzaud (1986) provides a nice review of enzyme methods as proxies for metabolism up to the time of the article's publication.

Successful application of enzymatic methods to estimate metabolic rates of zooplankton relies on a constant relationship between the two. Packard (1985) calculated a theoretical value of 0.5 for the ratio of respiration rate to ETS activity (R/ETS), assuming that Michaelis–Menten kinetics could be applied to respiratory chemistry and that the concentration of the respiratory regulator (ADP) was maintained near the Michaelis constant (K_m). The R/ETS ratios determined on size-fractionated mixed zooplankton from diverse oceanic systems at various seasons of the year by Hernández-León and Gómez (1996) are shown in Figure 10.4. It is clear that the ratio varies from near 0 to 2.8 with its mode around 0.5. The R/ETS ratios of marine zooplankton are known to be independent of temperature and little affected by body size (King and Packard 1975).

Much less work has been done on the ratio of GDH to ammonia excretion (GDH/NH_4). Bidigare and King (1981) showed a close relationship between GDH activities and ammonia excretion rates ($r = 0.92$, $n = 7$), which was confirmed by Park (1986a) ($r = 0.94$, $n = 5$) and Park *et al.* (1986) ($r = 0.98$, $n = 10$). However, Park (1986b) presented data on the variability of GDH/NH_4 for well fed and starved zooplankton. The GDH/NH_4 ratios determined on size-fractionated mixed zooplankton from waters

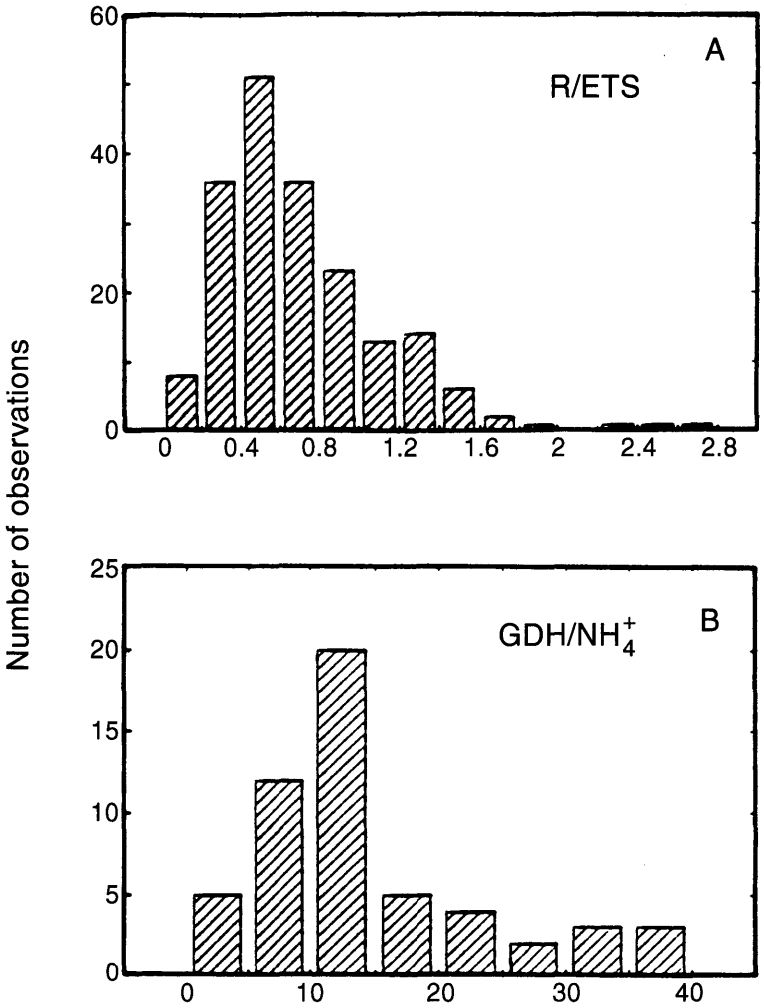

Fig. 10.4 Variations in the metabolic rate and enzyme activity ratio. (A) Ratio of respiration rate to ETS activity (R/ETS, $N = 202$) at experimental temperatures of 0.2–28 °C (redrawn from Hernández-León and Gómez 1996). (B) Ratio of GDH to ammonia excretion rate (GDH/NH_4, $N = 54$) at experimental temperatures 18–22.4 °C (Hernández-León and Torres 1997, with permission).

around Gran Canaria Island in spring and winter seasons by Hernández-León and Torres (1997) are summarized in Figure 10.4. It is apparent that, like the R/ETS ratio, the GDH/NH_4 ratios are also variable with a mode between 10 and 15. At present, accuracy of enzymatic methods to predict metabolic rates is seriously limited by inherent variabilities in the relationships with metabolic rates.

10.4.1 ETS activity

Enzymatic reaction rates follow Michaelis–Menten kinetics when substrate concentrations limit the rate of reaction. When enzymatic activity is measured, we are indirectly

measuring the amount of enzyme because activity is obtained at saturating substrate, i.e., at V_{max}. In contrast, we measure the metabolic rate *in vivo* in whole organisms whose cells and mitochondria may be subject to substrate limitation. If the enzymatic activity is only measured at V_{max}, a high variability in the ratio between metabolic rate and enzyme activity must be expected. This variability is found in the R/ETS ratio and Packard's argument invoking Michaelis–Menten kinetics, as stated above, has been used to explain it. This would be a biochemical explanation but a physiological one must also be sought.

Respiration rates of the copepod *Acartia tonsa* increased with increasing ration to a level three to four times standard metabolism (Kiørboe *et al.* 1985), the variability normally observed in the R/ETS ratio. It may be that, for a given ETS activity, a copepod's metabolism can vary from routine or active metabolism (high R/ETS) to standard metabolism (low R/ETS). Thus, ETS activity measures the potential of organisms to use their biochemical machinery to meet the physiological demands associated with different levels of activity. If physiological demands exceed the biochemical potential, the cells may adapt to the new situation by *de novo* synthesis of enzymes to increase the activity of the ETS. This would explain part of the variability in the R/ETS ratios found by Hernández-León and Gómez (1996) (cf. Figure 10.4). It has been recently observed (Hernández-León and Gómez 1996) that despite the possible variations in the size structure of zooplankton the R/ETS ratio of zooplankton varies with chlorophyl and primary production during development of the late winter bloom in the Canary Islands. They found higher R/ETS ratios coinciding with higher values of autotrophic production. Clearly, the organisms showed higher respiration rates coinciding with better food availability in quantity and/or quality. These results led Hernández-León and Gómez to believe that animals have a characteristic enzymatic level and that the respiration process does not use all the enzymatic capacity of cells (the Michaelis–Menten argument of Packard 1985). Animals can suddenly increase the locomotory activity or ingestion rate (and therefore the specific dynamic action) without changing the ETS activity level.

To test the Michaelis–Menten argument of Packard (1985), Hernández-León and Gómez (1996) designed an experiment using a flow-through system in which respiration rates and ETS activities of the copepod, *Calanus finmarchicus*, were measured simultaneously. Respiration rate was parallel to ETS activity (Figure 10.5B) when the copepods were fed synchronously. Increased respiration rates and ETS activities lasted only 4 h. They explained the response as respiratory control. Respiration in the mitochondria, the activity of its enzymes and the velocity of ATP production are driven by the relative concentrations of ADP, ATP and phosphate in the mitochondria and not by the

Fig. 10.5 Comparison of ETS activity and respiration rate in *Calanus finmarchicus* using a flow-through system. The experiment was run at 6.5 °C. (A) Respiration rate (open circles) and ETS activity (closed circles) of specimens under a continuous food supply. (B) Respiration rate and ETS activity of specimens provided low (LF) and high (HF) concentrations of food. The black bar indicates the dark period coinciding with the natural light–dark cycle. It is noted that a strong parallelism is seen between respiration rate and ETS activity. (C) Relationship between respiration rate and ETS activity from the experiments of (A) and (B). Points 1 and 2 in (C) are uncoupled data as indicated by an arrow in (A) and the first value of the same experiment. Units in (C) as in (A) and (B). Redrawn from Hernández-León and Gómez (1996, with permission).

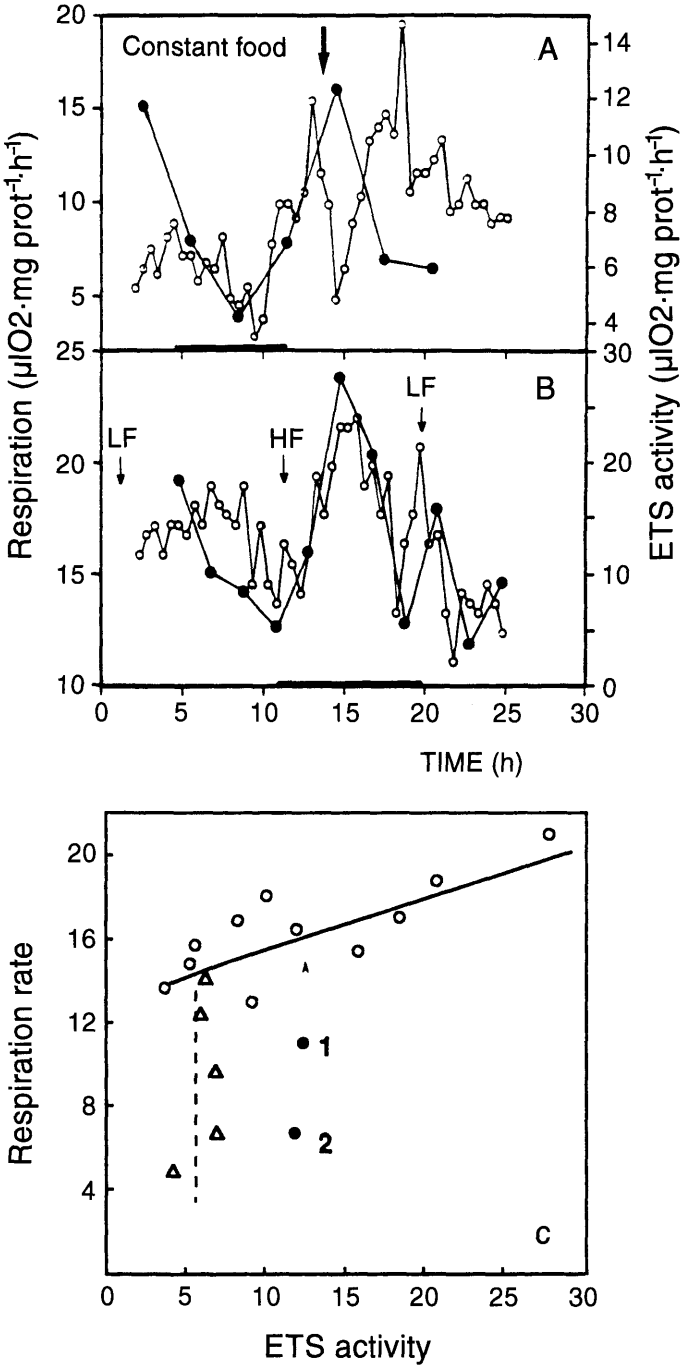
Constant food
A
B
LF
HF
LF
Respiration (µlO2·mg prot-1·h-1)
ETS activity (µlO2·mg prot-1·h-1)
TIME (h)
Respiration rate
ETS activity
1
2
C

Fig. 10.5

concentration of the respiratory substrates. When substrates are not limiting, the ADP and phosphate concentrations are high, and ATP is low, the oxygen consumption rate reaches its maximum. They suggested that ETS activity is measuring respiration when there is no substrate limitation (continuous line in Figure 10.5C). At low food concentrations, or when the copepods are incubated in filtered sea water, substrate limitation can be observed and then respiration is not predictable from ETS because the latter represents V_{max} (dashed line in Figure 10.5C). When an enzyme has a regulatory role, there is variability in K_m and therefore this parameter cannot be taken as constant. It is now evident that because of the variable nature of both enzymatic activity and metabolic rate on short time scales, search for a single conversion factor between the two variables does not make sense.

The coupling between respiration rate and ETS activity is corroborated by the results of Båmstedt (1980), Ikeda and Skjoldal (1980) and Skjoldal *et al.* (1984) who found ETS activities and respiration rates decrease in parallel during starvation. Finlay *et al.* (1983) also observed that respiration rates and ETS activities of protozoans vary in parallel except during starvation and re-feeding experiments. Recently, Packard *et al.* (1996) developed a bisubstrate enzyme kinetics model which enables the estimation of *in vivo* ETS activity for the marine bacterium *Pseudomonas nautica*. *In vivo* ETS activity thus estimated tracked well with respiration rate throughout all growth phases (including nutrient-starved senescent phase) of the bacterium. To estimate *in vivo* ETS activity using this bisubstrate enzyme kinetics model, intracellular substrates (nicotinamid adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH)) were determined, in addition to conventional *in vitro* ETS activity.

10.4.2 Enzymes of intermediary metabolism

Two assumptions underlie the use of metabolic enzymes as physiological indicators. The first is whether or not the concentration of enzymes within the aerobic and anaerobic pathways is modulated up or down by the cell in accordance with the cell's energy requirements, which dictates the carbon flow through those pathways. The second is that activity of the chosen enzyme accurately represents the activity of the pathway it is derived from. As with the ETS measurements, activities are measured under saturating conditions of substrate and cofactors so that what is obtained is an estimate of V_{max}. Comparisons of activities, or V_{max}, between individuals give good indication of differences in enzyme concentration, and therefore differences in physiological condition or metabolic poise (Hochachka and Somero 1984). A useful reference that discusses the rationale for choosing V_{max} as a basis for comparison is Newsholme and Crabtree (1986).

Use of intermediary metabolic activities (as distinguished from the ETS assay treated in ETS Activity, section 10.4.1) as indicators of physiological status, or as proxies for metabolism, is fairly new to zooplankton biology. With a few exceptions (e.g. Curl and Sandberg 1961; Packard and Taylor 1968) all the published work dates from 1990. However, much groundbreaking had already been done with fishes (Childress and Somero 1979; Somero and Childress 1980; Sullivan and Somero 1983; Kaupp 1987; Lowery *et al.* 1987; Torres and Somero 1988; Kaupp and Somero 1989) so the fact that metabolic enzyme activities had good potential as physiological indicators was already known; what was required was the transfer of the techniques to planktonic animals.

Clarke *et al.* (1992) described the influence of ration level on activities of LDH and CS in two species of very young fish larvae (ichthyoplankton): red drum (*Sciaenops ocellatus*)

and lane snapper (*Lutjanus synagris*). It was found that growth in the ichthyoplankton correlated quite positively with activities of LDH and CS. A similar result was obtained for the copepods *Acartia tonsa* and *Temora longicornis*; copepods starved over a 36 h period showed a significant drop in CS activity when compared with well-fed controls. The results of Clarke and Walsh (1993) echo much of the early work on succinate dehydrogenase (SDH) in zooplankton in which SDH activity was observed to correlate well with oxygen consumption rate in *Artemia* (Packard and Taylor 1968) and with seasonal changes in condition in the mysid *Neomysis integer* (Raymont *et al.* 1967).

Recent work by Thuesen and Childress (1993a, 1993b, 1994) has greatly expanded the phyletic range in which intermediary metabolic enzyme activities (CS, MDH, LDH, PK) have been examined. Their work describes enzyme activities and metabolism in nemerteans, annelids, chaetognaths and cnidarians. For our purpose, it is important on two main fronts. First, it shows that enzyme activities can be measured readily even in the very watery (> 95% of wet weight) gelatinous zooplankton. Second, there is a strong correlation of oxygen consumption, enzyme activity, and life habit in the zooplankton examined. Scaling coefficients ('*b*') for aerobic enzymes were species-specific and varied between 0.67 and 1.0.

Intermediary metabolic enzymes show excellent promise as indicators of condition and as proxies for metabolic rate. However, no published work has attempted to correlate intermediary metabolic enzyme activities and short-term, biologically important, environmental characteristics such as primary production or chlorophyl biomass. Preliminary data generated by one of the author's (J.J. Torres) laboratories during an Antarctic field study fills this need in addition to strongly supporting the use of metabolic enzymes as indicators of condition of copepods. Adult *Rhincalanus gigas*, a dominant copepod in the Antarctic system, were collected during the annual spring bloom of phytoplankton at the edge of the retreating pack-ice in the Scotia–Weddell Sea region. Three locations were sampled: a low productivity area deep within the pack-ice, the highly productive area within the ice-edge bloom, and the less intense, but still productive area seaward of the bloom. Oxygen consumption rates were measured at sea (Figure 10.6A) and CS activities were determined in the laboratory (Figure 10.6B) on *R. gigas*, the latter were frozen in liquid nitrogen and maintained continuously at $-80\,^{\circ}C$. There was a significant difference ($P < 0.05$, ANOVA) in oxygen consumption rate and in CS activities between the low productivity pack-ice zone (zone 5) and the higher productivity ice edge (zone 3) and open water stations (zone 1). The fact that oxygen consumption and CS activity covary with chlorophyl biomass suggest first, that CS has considerable promise as a biochemical proxy for metabolism, and second, that it is responsive to conditions of the copepod in the field.

10.4.3 Potential sources of error

In addition to the quality and quantity of food in a zooplankter's diet (see Feeding, section 10.3.1) several other variables may give rise to errors in enzymatic estimation of metabolic rate.

GROWTH CONDITIONS

Little information is available for zooplankton describing the influence of growth conditions on the relationship between enzyme activity and metabolic rate. A higher R/ETS ratio associated with active growth and a lower R/ETS ratio associated with senescence have been observed in bacteria (Christensen *et al.* 1980) and microalgae

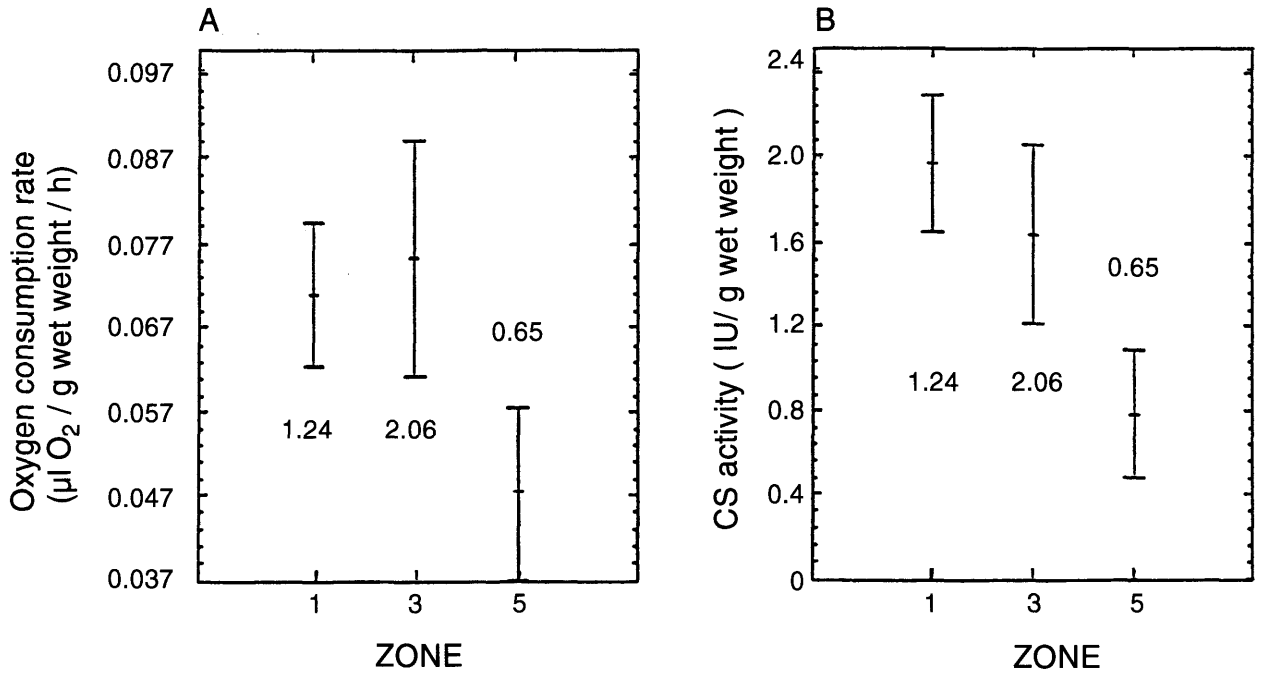

Fig. 10.6 Oxygen consumption rate and citrate synthase (CS) activity, the enzyme catalyzing the initial step in the Krebs Cycle, of the Antarctic copepod, *Rhincalanus gigas*, with relation to the Antarctic ice edge. Zone 5 is consolidated pack-ice, zone 3 is the highly productive marginal ice zone, zone 1 is the open water seaward of the ice-edge bloom. Oxygen consumption rates are expressed as μl O_2 g wet weight^{-1} h^{-1}. Enzyme activity is expressed in international units per gram wet weight (IU g^{-1}: IU = μmol substrate converted to product per minute). Error bars are 95% confidence limits. Numbers within the figure are the values for chlorophyl biomass (μg l^{-1}) corresponding to each zone. Oxygen consumption rates were significantly ($P < 0.05$; ANOVA) lower in the pack ice (zone 5) than at the ice-edge (zone 3) or open water (zone 1) locations. CS activities were significantly ($P < 0.05$; ANOVA) lower in the pack ice (zone 5) than at the ice-edge (zone 3) or open water (zone 1) locations. Oxygen consumption data contributed by Helena Kawall, and CS activity data by Steve Geiger.

(Kenner and Ahmed 1975). As mentioned in Enzymes of intermediary metabolism, section 10.4.2, Clarke *et al.* (1992) found that growth of ichthyoplankton (larvae of *Sciaenops ocellatus* and *Lutjanus synagris*) correlated well with activities of LDH and CS. In contrast, Berges *et al.* (1993) observed that activities of CS and GDH did not correlate well with specific growth rate in laboratory-raised *Artemia franciscana* and cautioned against their use in the field. These inconsistent results may reflect the physiological dissimilarities of experimental organisms between studies, but evaluation of growth conditions may be a necessary adjunct for obtaining reliable correlations between metabolism and biochemical proxies in field-caught plankton.

BODY SIZE

A series of two papers (Berges *et al.* 1990; Berges and Ballantyne 1991) described scaling of metabolic enzyme activities with body size at different ration levels and in different species (*Artemia franciscana, Daphnia magna*). The authors concluded that scaling with body size was enzyme dependent and often differed from the classic '*b*-value' of 0.75 in the allometric equation between metabolic rate (M) and body mass (W) (i.e. $M = aW^b$, where a is a constant, see also Body size and temperature as bases of metabolic comparison, section 10.3.4, and equation 10.5), hence scaling must be considered in any field application of enzyme activities as biochemical proxies.

A large amount of scatter in the R/ETS and GDH/NH_4 data in Figure 10.4 may be due to the mixture of zooplankton with different body sizes (= body mass). Christensen *et al.* (1980) compared R/ETS ratios of various marine organisms including bacteria to zooplankton (body mass: 10^{-7} to 10^3 mg dry weight) and noted only a slight dependence of the ratio on the body mass (mg DW) of organisms:

$$\log_{10}(\text{R/ETS}) = -0.0485 \log_{10}\text{DW} + 0.191. \qquad (10.9)$$

Packard (1985) summarized the R/ETS ratio to be 0.17 for phytoplankton, 1.1 for bacteria, 0.25 for protista and 0.49 for zooplankton. Analyzing the relationship between respiration rates and ETS activities, a linear regression, instead of a mean, has been suggested to improve the precision in estimation of the former from the latter (King and Packard 1975; Arístegui and Montero 1995).

MISMATCH BETWEEN METABOLIC RATE AND ENZYME ACTIVITY

Båmstedt (1980) noted a delayed response of ETS activities to increased respiration rates in *Acartia tonsa*. Finlay *et al.* (1983) found a time-lag between respiration rates and ETS activities on ciliates which were starved and then fed. Finlay *et al.* explained the cause of the time-lag as a limitation in the amount of enzymes involved in the ETS. This uncoupling would be a source of variation in the relationships between enzymatic activities and metabolic rates of organisms collected in the field, where food availability may change over short time scales as a consequence of physical instabilities of water columns. The time-lag observed by Finlay *et al.* (1983) on ciliates was on an hourly time scale. While the application is presently limited to the bacteria bisubstrate enzyme kinetics model proposed by Packard *et al.* (1996) should provide a better insight to the uncoupling problems between respiration and ETS activity.

CALIBRATION DESIGN

Present calibration procedures for the relationship between enzyme activity and metabolic rate appear inadequate. Figure 10.7 illustrates a typical calibration of ETS activities against respiration rates using the sealed chamber method as practiced by

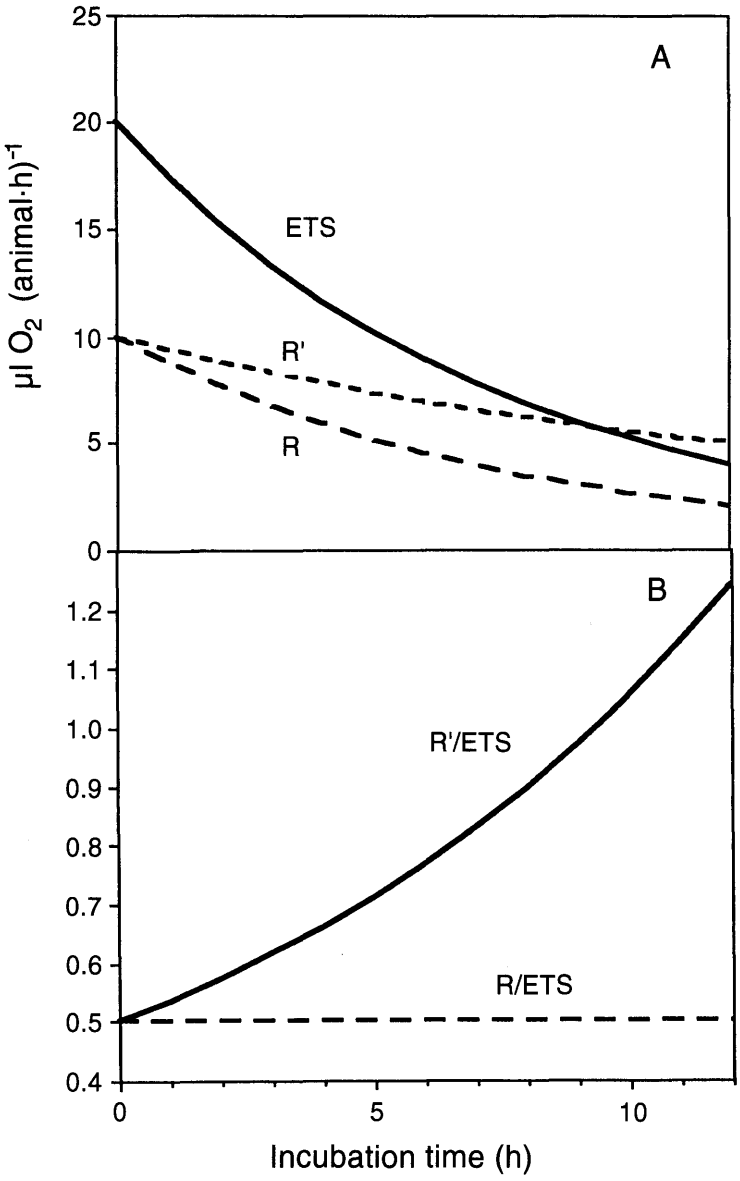

Fig. 10.7 (A) Model run for the likely changes in the ETS activity and respiration rate (R, and its integrated mean over incubation time R′) of zooplankton placed in a closed bottle filled with filtered sea water. (B) Resultant R/ETS (and R′/ETS) ratio. In this run, ETS and R at the start ($t = 0$) is designated as 20 and 10 μl O_2 (animal h)$^{-1}$, respectively, and decrease exponentially to 4 and 2 μl O_2 (animal h)$^{-1}$, respectively, at the end of incubation lasting for 12 h, maintaining a constant R/ETS ratio of 0.5. Note that the decrease with the progress of incubation time of R′ is much slower than that of R, which reflects an increase of R′/ETS during the incubation. Assumed equations for the change with time are; ETS = $20e^{-0.1341t}$, $R = 10e^{-0.1341t}$, and $R' = \int_0^t R\,dt = 10(1 - e^{-0.1341t})/(0.1341t)$.

previous workers (Båmstedt 1980; Ikeda and Skjoldal 1980; Skjoldal *et al.* 1984). In the sealed chamber method, respiration rate is determined from the difference in the oxygen concentration between experimental and control bottles at the end of the incubation (i.e. R′: integrated mean respiration rate over the incubation period in Figure 10.7), and ETS activity is measured on zooplankton retrieved from experimental bottles at the end of incubation. Because of this methodological constraint, the R′/ETS ratio obtained from the sealed chamber method is subjected to error unless there is a constant respiration rate and ETS activity over the incubation period. In the model shown in Figure 10.7, a rapid decrease of ETS activity and a less pronounced decrease of integrated mean R′ reflected a progressive increase of the R′/ETS (1.24 at the end of 12 h, in contrast to a designated ratio of 0.5). The problem highlighted in the model is not limited to the ETS activity, it applies to the activity of other enzyme systems as well. To overcome this problem, a continuous recording system for metabolic rate, such as the flow-through system (Measuring metabolic rate on live zooplankton, section 10.3) is more appropriate for the purpose of calibration.

10.5 CONCLUDING REMARKS

While the determination of metabolic rates of live zooplankton is easily accomplished with either the sealed chamber method or the flow-through method, extrapolation of measured rates to a species population in the field is not straightforward. One obvious problem is the lack of a clear index to judge whether or not the specimens collected from the field and brought into the laboratory are 'damaged'. Part of the problem may be overcome by experience and by checking morphology, coloration, swimming behavior, orientation patterns, and response to stimuli (light, touching with needle, etc.). However, this sort of quick screening prior to an experiment may not always detect physiological damage.

The second problem associated with experiments on live zooplankton is the difficulty in reproducing natural environmental conditions for zooplankton in a laboratory experiment. Environmental and nutritional conditions of zooplankton in the laboratory affect the metabolism of zooplankton. Our knowledge about the quantitative metabolic responses of zooplankton to environmental and nutritional conditions is quite limited. Most previous studies concern one or two potential parameters affecting metabolism; few attempts have been made to examine the combined effects of various parameters. Newell *et al.* (1976) analyzed a combination of six variables (exposure temperature, body weight, starvation period, feeding period, acclimation temperature, and lipid content) on the oxygen consumption rate of an intertidal isopod, *Ligia oceanica*, in the laboratory. They found that 85% of the observed variation in the rates could be attributed to the six parameters. An approach of this sort is of great importance for the extrapolation of laboratory results to field populations of zooplankton.

The third problem is the lack of adequate techniques to control swimming activity of test zooplankton during experiments. It has been assumed that the change in swimming activity of animals brought in the laboratory and confined in containers for metabolic measurements is minimal. This may be the case for small, less active zooplankton. For larger and more active zooplankton such as euphausiids, determination of metabolic rate under well defined activity levels of animals (i.e. standard, routine, active metabolism) is needed for valid comparison of the data between workers and for better estimation of metabolic costs for diel vertical migration in the field (Torres and Childress 1983).

The fourth problem is associated with zooplankton themselves; an assemblage composed of highly diverse groups of animals (11 phyla at least, cf. Omori and Ikeda 1984) poses a real problem in establishing standardized methods for measuring their metabolic rates. High phylogenetic diversities are inevitably accompanied by a wide body-size range, and dissimilarities in behavior, locomotory activity, physiology and nutrition of species are involved. It is almost certain that inconsistent experimental results on zooplankton between workers reflect the complexities of the animal groups they have studied.

Contrary to earlier expectations, enzymatic methods for estimating metabolism are not very simple, and are not necessarily free from the problems discussed here. In addition to requirements for proper calibration with metabolic rate measurements on live zooplankton under controlled laboratory conditions, information about nutritional state, growth condition and size of animals may also be required for the better estimation of metabolic rates of zooplankton in the field. In this regard, direct determination (sealed chamber method or flow-through methods) and indirect determination (enzymatic methods) will complement each other as we work toward establishing better methodologies for assessing zooplankton metabolism.

Practice (T. Ikeda and J.J. Torres)

10.6 COLLECTION AND HANDLING OF ZOOPLANKTON

Most zooplankters are fragile, and for use in metabolic experiments it is important that the specimens remain undamaged. Figure 10.8 shows two examples of collecting buckets designed for such samples (see also Reeve 1981), their volumes (2 to 30 l) are several times larger than that of an ordinary cod-end bucket and they have mesh windows in the anterior part.

The cod-end bucket is filled with sea water prior to sampling so that the net will sink promptly when deployed. A vertical haul or short horizontal tow with a fine-mesh net is preferable in order to avoid damage to the plankton during the haul. The towing speed

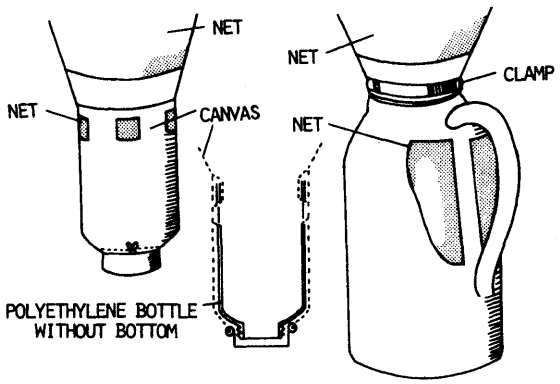

Fig. 10.8 Two examples of cod-ends for collecting live zooplankton (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

should be about 0.3 m s^{-1} or less, and when sampling from a large ship, it is better to stop the engine and make use of the drift of the ship caused by the wind. A long tow weakens the plankton in the net. In tropical waters, where the water temperature is high, very short tows should be used. After retrieving the net, the bucket is quickly placed in a container filled with a large amount of sea water, after which the samples are gently transferred to the container. The plankton that remain attached to the net should be considered damaged and not transferred into the container. As plankton are damaged from direct exposure to the sun, the best sampling time is after sunset (Light, section 10.3.1).

To sample actively swimming macroplankton, a net with a large mouth and good filtration efficiency, such as the ORI-C net (Omori 1965), should be used. The posterior part of the ORI-C net is made of mesh gauze which is finer than the anterior section to minimize damage to the organisms during the tow. Additional protection for specimens may be achieved by using specialized cod-end buckets that are designed to close at the end of the tow, thereby creating a parcel of protected water for bringing animals to the surface unharmed. For example, the thermal cod-end described by Childress *et al.* (1978) has been used to successfully recover deep-living oceanic zooplankton. Johnson and Attramadal (1982) devised another type of protective cod-end bucket for use on sledges to collect epibenthic animals. This simply designed bucket can be modified for midwater horizontal tows to avoid the mechanical damage of specimens and prevent exposure to large fluctuations in temperature, salinity, and light during recovery.

Gelatinous plankton are best captured by divers who introduce them into wide-mouth containers, but some may be obtained with net hauls in the manner described above. To transfer specimens to another container, they should be scooped gently with a small beaker or specially designed net (Figure 10.9A).

When transporting the living zooplankton to the laboratory, their density should be kept as low as possible. Polyethylene containers with airtight lids are excellent for this purpose. To reduce damage of organisms due to agitation of water during transport, the container should be completely filled. Also, every effort should be made to avoid direct sunlight and change in water temperature. It is therefore advisable to cover the containers with dark cloth. For certain species aeration (gentle bubbling of sea water with air from air compressors) may be needed if transportation takes a long time.

For sorting specimens, a portion of the sample is gently poured into a transparent container of appropriate size and healthy animals are removed by pipette, spoon or scoop. The pipettes are made of glass or clear acrylic tubes of various diameters. Simple apparatus and methods to aid sorting of live zooplankton are shown in Figure 10.9 (B and C). In some cases the sample may be left for 10 to 30 min after having been transferred to a container, and the differences in the phototaxis or geotaxis of different species used to good advantage in sorting. Small transparent organisms are easily seen if the background of the container is darkened and light enters through the side. For some species, their characteristic swimming behavior as well as body coloration may distinguish them for sorting. Selection of experimental specimens is based on appearance. Any evidence of physical damage such as bruising or opaque patches on an otherwise transparent individual constitutes grounds for rejection. With crustaceans, any fouling of limbs or unnaturally limited range of motion on the swimming legs would be a basis of rejection. It is worth the energy to observe living zooplankton in beakers, buckets, or petri-dishes to get a feeling for how they move and what a normal range of limb motion is. There is no substitute for knowing your animal!

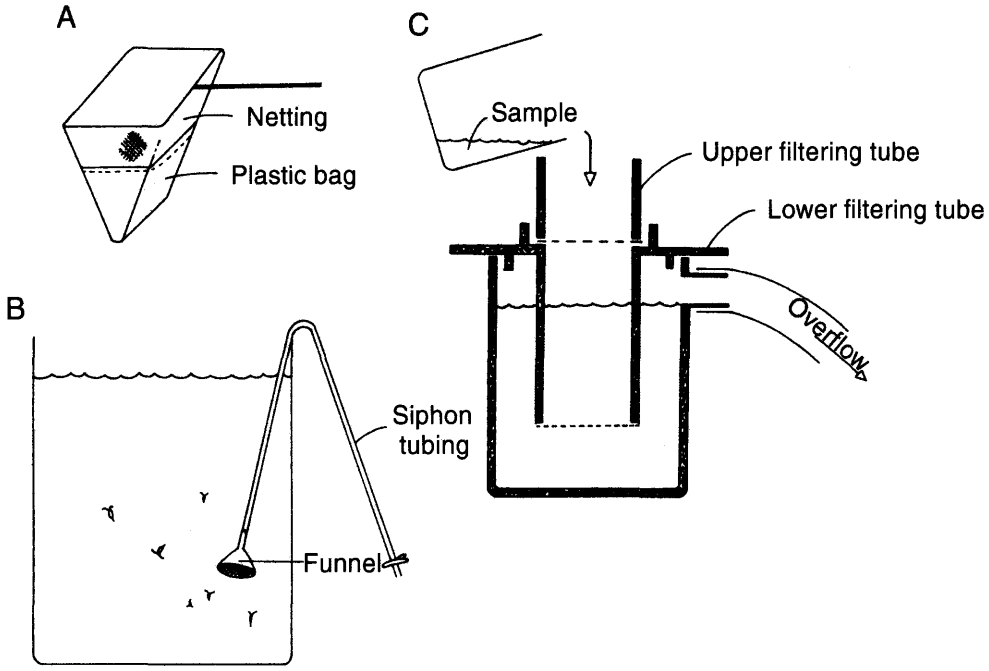

Fig. 10.9 Simple apparatus to aid sorting of living zooplankton. (A) net of which the lower part is replaced with a plastic bag. With this net large zooplankton are transferred with a certain amount of water from one container to the next without exposing them to air. (B) A siphon system to remove unwanted small specimens. On one end of the system is fitted a funnel covered with a mesh screen. A gentle siphoning out of the water is essential to avoid damaging larger specimens. (C) A double filtering system to remove undesired larger and smaller zooplankton simultaneously. A zooplankton sample is gently poured into the upper filtering tube through a coarse mesh screen and passes into the lower filtering tube through a fine mesh screen into a container filled with sea water. The desired intermediate size zooplankton are retained in the lower filtering tube. Upon completion, the upper filtering tube is removed and zooplankton in the lower tube are transferred to the container with a pipette. A whole set is made with Plexiglass or Perspex (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

With regard to the time delay between actual capture of zooplankters and subsequent measurements, there is a trade-off between stress and starvation (Capture stress/starvation, section 10.3.1). Specimens should be allowed to recover from the trauma of capture for a period of time but not for so long that the metabolism drops significantly due to starvation. Different investigators use different recovery periods varying from 2 h to greater than 24 h. A few things should be kept clearly in mind when deciding for oneself how long to allow animals to remain in the unnatural environment of the laboratory before using them in experiments. First, any manipulation of the experimental subject is a potential source of stress or excitement. If the investigator is concerned with accumulation of oxygen debt during capture, similar concern should be extended after the introduction into the respirometer. Second, the animal is never going

to be healthier and happier than it was when swimming in the ocean. Even if it can be maintained in the laboratory and fed for days or weeks it is unlikely in the extreme that it is improving in health. Thus, in principle, the sooner after capture that a metabolic rate is taken, the better the result is likely to be.

10.7 RESPIRATION

10.7.1 Oxygen consumption – Winkler titration (T. Ikeda)

The method described below largely follows Omori and Ikeda (1984). In the sealed chamber method, bottles with and without experimental zooplankters are prepared simultaneously and the difference in concentrations of dissolved oxygen after a period of incubation is attributed to respiration of the zooplankters. This method has been the most popular since the original oxygen consumption measurements on *Calanus finmarchicus* by Marshall *et al.* (1935).

The major advantage of the water bottle method is its simplicity in that it requires no specialized equipment. However, the method requires relatively long incubations, therefore starvation cannot be completely eliminated as a potential influence on the rate (Technical problems, section 10.3.1). Since the changes in oxygen consumption rate of zooplankton during the incubation period cannot be determined, the method assumes that the oxygen consumption rates of zooplankters are constant during the entire incubation period.

INCUBATION BOTTLES

BOD bottles are ideal, but ordinary glass reagent bottles fitted with airtight lids may also be used. The capacity of each bottle should be measured prior to its use in an experiment. The capacity may be obtained from the difference in the weight of each bottle with lids before and after filling it with distilled water (1.00 g = 1.00 ml). Prior to the experiment bottles and pipettes for the transfer of specimens from one container to the other should be cleaned with acid solution (6*N* HCl), then rinsed well with pure water and dried.

WASHING/EXPERIMENTAL SEA WATER

Water sampled from the site from which zooplankters were obtained is used to wash and subsequently incubate the zooplankters. In order to remove other organisms and particles, water should be filtered through GF/C, GF/F or Nuclepore filters with equivalent pore-size (0.4 μm). To remove smaller oceanic bacteria, Nuclepore filters with 0.2 μm are best. Vacuum filtration usually reduces the dissolved oxygen content in water. Therefore, aeration of the water is necessary to readjust the oxygen content to near saturation level. Most experiments start at oxygen saturation. Special attention may be required for experimentation on animals from low oxygen habitats. To remove particles from low-oxygen water without appreciable changes in oxygen content, a gentle filtration system (see Figure 10.9B) using a combination of a siphon system and funnel covered with fine mesh netting (10 or 20 μm mesh openings) may be used.

Three typical washing procedures are illustrated in Figure 10.10 A–C. The best washing method depends on the character of the zooplankters and the experimental design. Generally, the washing procedure is not so critical for oxygen consumption rate measurements with oxygen saturated water, but it is a very critical source of error for

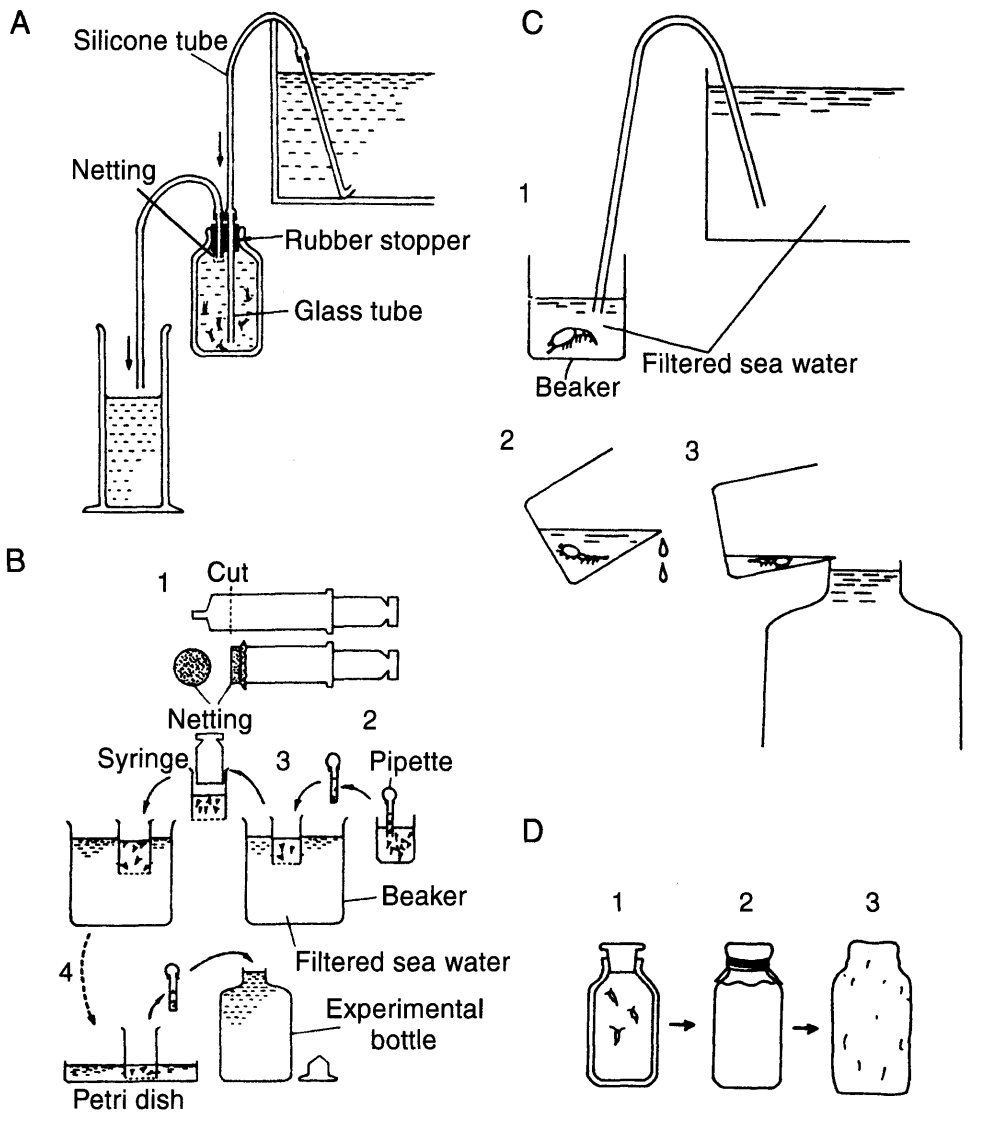

Fig. 10.10 Three procedures for washing zooplankton for metabolic experiments, and preparation of bottles for incubation after washing. (A) A siphon system, originally designed by Marshall *et al.* (1935) for oxygen consumption measurements. In their original method, sea water volume needed for flushing is 6–7 times the bottle volume. (B) Transparent plastic syringe. Place animals in the syringe with a pipette, and rinse them in several beakers. Then, place the syringe into a petri-dish and introduce the animals into bottle with pipette. A convenient size of syringe is 20 ml (2 cm in diameter), but larger ones can be used when fine netting is placed over the tip. (C) Glass beaker for relatively large zooplankton. Gently add filtered sea water in the beaker and decant carefully. Repeat this treatment 3 to 4 times, and then place the animals into a bottle. (D) Preparation of a bottle for incubation. See text (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

ammonia and phosphorus excretion measurements mentioned in section 10.8. It is very important not to damage the specimens while washing them. The number of zooplankters should be counted before incubation (and again at the end of incubation). The bottles are filled with experimental water using a siphon system, placing one end of the siphon onto the bottom of the bottles. After zooplankters have been washed and placed into the bottles, lids should be fitted so as to avoid trapping any air bubbles. The lids are firmly wrapped with a plastic sheet and rubber band to reduce the risk of introduction of air bubbles in the bottles during incubation. Control bottles without zooplankters should be prepared concurrently using exactly the same procedure. In a typical experiment with ten experimental bottles, two control bottles are prepared before the first experimental bottle and two after the last experimental bottle. An additional control bottle may be inserted between the fifth and sixth experimental bottles (15 bottles in total). The bottles are ready for incubation after wrapping with aluminum foil or black plastic sheet (Figure 10.10D). A water bath with a temperature control unit is best for incubating the bottles.

INCUBATION

The incubation period will vary depending on the experimental temperature, density of zooplankters relative to the volume of the bottle, and rates of oxygen consumption of the zooplankters. For medium to large sized zooplankters, 12 or 24 h is recommended to eliminate the effect of diurnal rhythm on the metabolic rates. The period may be shortened to only a few hours for very active zooplankters that are less tolerant to prolonged starvation during the incubation period. The length of incubation period should first be based on the size of zooplankton (i.e. tolerance to starvation). Decision on the size of bottles used in the incubation and the number of specimens in each should be based on the need to minimize accumulation of excreta and oxygen depletion at the end of the incubation (see Oxygen saturation, section 10.3.1).

Antibiotics, such as streptomycin and chloromycetin, are often used to minimize bacterial activity in the water. The difficulty with using antibiotics is the determination of dosages, which vary among different zooplankters. Unfortunately, little is known about the appropriate dose of antibiotics for different zooplankton, and excess antibiotics may seriously affect the activity of experimental animals. Some antibiotics may interfere with other chemical analyses (Bacteria, section 10.3.1).

To determine dissolved oxygen, a volume of water is removed from the bottle after incubation. Figure 10.11 shows a siphon system that we use to transfer water from incubation bottles into small oxygen bottles. Duplicate water samples are sufficient when the analyst is skilled in the technique.

At the end of the incubation, the activity of zooplankters in the experimental bottles should be checked. These zooplankters may then be transferred to a petri-dish (larger zooplankters) or directly onto a piece of mesh (smaller zooplankters). Specimens can be counted either with the unaided eye or, for smaller zooplankters under a dissecting microscope. Zooplankters should then be weighed (wet weight) and/or weighed after drying (dry weight). Prior to weighing, water adhering to the body of a specimen should be removed by placing the specimen on filter paper. For smaller zooplankters that are difficult to handle individually, this can be achieved by placing the filter paper on the other side of the mesh. Although a brief rinse with distilled water to remove salts is desirable it is sometimes accompanied by loss of integrity, and organic matter, due to body lysis from osmotic shock (see Omori 1978).

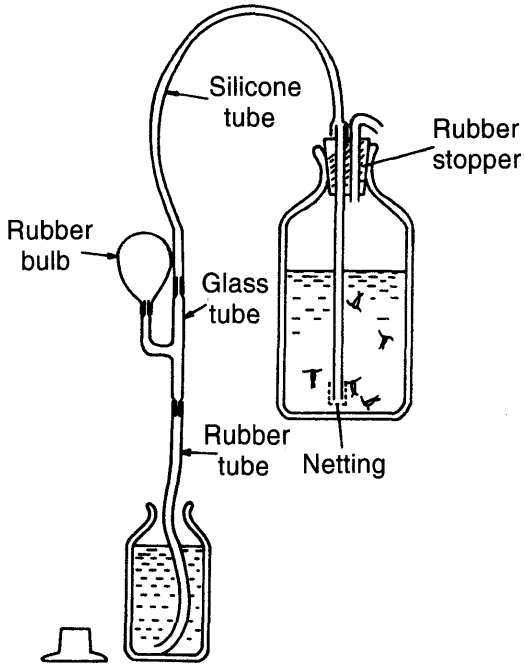

Fig. 10.11 A siphon system to transfer sample water from experimental bottles into oxygen bottles at the end of incubation (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

TITRATION

Numerous minor modifications have been made for the original titration method of Winkler (1888), and the following is one by Strickland and Parsons (1972).

Analytical apparatus

300-ml BOD bottles, 10-ml titration burette (with 0.05-ml scale divisions), 1-ml pipette (dispensing type), 50-ml pipette, 125–250-ml conical flasks or beakers (it is easier to discriminate between the colors during titration if the entire bottom and half of the sides are painted white on the outside), As a titration burette, the Metrohm piston burette (cylinder volume: 10 ml) is accurate and easy to handle, especially for shipboard measurements.

Reagents

1) Manganous sulfate reagent: 480 g of manganous sulfate tetrahydrate $MnSO_4 \cdot 2H_2O$ or 365 g of $MnSO_4 \cdot H_2O$) is diluted in distilled water to make a volume of 1 l.
2) Alkaline iodide solution: 500 ml of sodium hydroxide NaOH (analytical reagent grade) is dissolved in 500 ml of distilled water. 300 g of potassium iodide KI (analytical reagent grade) is dissolved in 450 ml of distilled water. Mix the two solutions together. A great deal of heat will be liberated.
3) Approximately 0.01*N* sodium thiosulfate: 2.9 g of high-grade sodium thiosulfate $Na_2S_2O_3 \cdot 5H_2O$ and 0.1 g of sodium carbonate Na_2CO_3 are dissolved in 1 l of distilled water and one drop of carbon disulfide CS_2 per liter is added as a

preservative. The solution should be prepared 24 h before use and is stable over a long period if stored below 25 °C in a dark well-stoppered bottle.

4) 0.0100*N* iodate solution: A small amount of potassium iodate KIO_3 (analytical reagent grade) is dried at 105 °C for 1 h and exactly 0.3567 g weighed out after cooling. This is dissolved in 200–300 ml of distilled water by warming slightly. After cooling, distilled water is added to make a solution of 1 l. The solution is stable indefinitely if kept in a brown bottle in a dark cool place.
5) Starch indicator solution: 0.1%–0.2% of soluble starch solution is used. For long-term use preservation solution can be made as follows: 2 g of soluble starch is suspended in 300–400 ml of distilled water and a 20% solution of sodium hydroxide is gradually added with vigorous stirring until it becomes clear. After 1 to 2 h concentrated hydrochloric acid is added until the solution becomes slightly more acidic to litmus paper. Then 2 ml of glacial acetic acid is added. Finally, distilled water is added to make a solution of 1 l.

Determination of the factor for the sodium thiosulfate solution

A 300-ml BOD bottle is filled with sample water, add 1.00 ml of concentrated sulfuric acid and 1.00 ml of alkaline iodide solution and mix thoroughly. Then 1 ml manganous sulfate solution is added and mixed well. A 50-ml aliquot is poured into each titration flask. Use one or two flasks for blank determinations. Exactly 5.00 ml of iodate solution is added with a 5-ml pipette. Allow iodine liberation to proceed for 2 to 5 min at a temperature below 25 °C and out of direct sunlight, then titrate the iodine with the thiosulfate solution. The starch indicator is added when the yellow color of the liberated iodine fades; the first moment of the disappearance of blue color is the end point. If V is the titration volume of sodium thiosulfate solution in milliliters, the calibration factor f is:

$$f = 5.00/V. \tag{10.10}$$

The titration should be performed with at least three flasks and the average value obtained becomes f. A blank determination is made by titrating without adding iodate solution. When the blank correction exceeds 0.1 ml of sodium thiosulfate, the reagents are suspect and should be made afresh.

Analytical procedure

1) Remove the stopper from the 300-ml BOD bottle containing the sample water, and, by placing the tip of a pipette a little below the water surface, add 1.0 ml of the manganous sulfate solution followed by 1.0 ml of alkaline iodide solution. Then carefully restopper the bottle immediately so that bubbles are not trapped in it and shake the bottle well. After 2 to 3 min, shake the bottle again. Then allow the BOD bottles to stand quietly for several hours at room temperature until the precipitate has settled at least one-third of the way down the bottle, leaving a clear supernatant solution. Immersion of the bottles into water is recommended to prevent introduction of air bubbles prior to titration.
2) The titration is performed within several hours to one day after fixation. Remove the stopper from the bottle and add 1.0 ml of concentrated sulfuric acid by placing the tip of a pipette just below the water surface. Then restopper the bottle and shake the bottle well in order to dissolve the precipitate.
3) Transfer 50.0 ml of this solution into the specially painted flasks or beakers by means of a pipette. Titrate immediately with standard 0.01*N* thiosulfate solution. During titration the water should be stirred by a magnetic stirrer. When the straw color of the iodine becomes very pale, add 5 ml of starch indicator. The titration is

continued until the blue color disappears; this moment is the end point. Read the volume of sodium thiosulfate used up to the end point.

If the volume of the BOD bottle is Y (ml) and that of sample water used for titration is X (ml) and the titration volume of $0.01N$ sodium thiosulfate solution is expressed as V (ml) and the blank correction as V' (ml), the dissolved oxygen concentration in the sample water can be obtained by the following formula:

$$\text{mg-at } O_2\, l^{-1} = Y/(Y - 2) \times 5.00/X \times f \times (V - V') \tag{10.11}$$

where 2 in the denominator $(Y - 2)$ of the first term of the right-hand side is the total volume of the manganous sulfate solution (1.0 ml) and alkaline iodide solution (1.0 ml) added.

Thus, when a 50-ml aliquot is taken from a 300-ml BOD bottle,

$$\text{mg-at } O_2\, l^{-1} = 0.1006 \times f \times (V - V')$$

The milliliters or milligrams of oxygen in a liter of water can be calculated from the expressions

$$\text{ml } O_2\, l^{-1} = \text{mg-at } O_2\, l^{-1} \times 11.2 \tag{10.12}$$

$$\text{mg } O_2\, l^{-1} = \text{mg-at } O_2\, l^{-1} \times 16.0 \tag{10.13}$$

Note: When BOD bottles or similar glass bottles less than 300 ml are used, the amounts of reagents may be reduced proportionally. As an example, if 30-ml bottles are used, the amounts of reagents are 1/10 these given above. The titration of the entire contents in a titration flask is recommended rather than taking aliquots. Use the following formula for calculating the dissolved oxygen concentration

$$\text{mg-at } O_2\, l^{-1} = 1/(Y - 0.2) \times 5.00 \times f \times (V - V') \tag{10.14}$$

The author (T. Ikeda) has applied this scaled-down Winkler method successfully to quantities of sample water as small as 5 ml (though some loss in precision is inevitable). For even smaller quantities of sample water (1–2 ml), the use of a special version of this method, the so-called micro-Winkler method, may be recommended (for details, see Barnes 1959).

CALCULATIONS

Oxygen consumption rate (R) may be calculated using the following equation:

$$\begin{aligned} R &= [(C_o - C_t) - (C_o - C_{t'})] \times (V_c - V_z)/[t \times (N \text{ or } W)] \\ &= C_t - C_{t'}) \times (V_c - V_z)/[t \times (N \text{ or } W)], \end{aligned} \tag{10.15}$$

where C_o is the oxygen concentration at the beginning of incubation; C_t and $C_{t'}$ are oxygen concentrations in control and experimental bottles, respectively, at the end of incubation; V_c is the volume of experimental bottles; V_z is the volume of zooplankters; t is the incubation time; N is the number of specimens, and W is the mass of specimens used. Note that C_o is canceled out in the calculation, it is therefore not necessary to determine it in the experiment in which filtered sea water is used. V_z is estimated from the wet weight of the zooplankters assuming 1 ml = 1 g wet weight, but this is negligible in most experiments.

R is expressed in two ways: per individual and per unit mass (mass-specific or, formerly more common, weight-specific rate) of zooplankters. For the purpose of this

manual we will use the terms mass and weight interchangeably. Specimen total mass is often not mentioned in publications where the mass-specific expression is used. In either expression of R, data on the mass of zooplankters should be provided with R so that other researchers can interconvert if necessary, but also because body mass has a considerable influence on metabolism (see Time-course method in section 10.9.1)

10.7.2 Oxygen consumption – electrodes (J.J. Torres)

Oxygen electrodes provide a valuable alternative to the Winkler technique for measuring dissolved oxygen. The basic principles of taking the respiratory determination remain the same as those used in bottle incubations, i.e. the experimental subject is confined to a known volume for a period of time determined by the researcher and, of course, the oxygen requirements of the organism. The advantage to the investigator using O_2 electrodes is that oxygen within the experimental vessel is continuously monitored. This is not a trivial matter, as it allows the investigator to monitor oxygen consumption with respect to other variables, for example, the animals' locomotory activity, or time after feeding, without the need for taking many sequential sub-samples of the volume of water the animals are residing in. In addition, since oxygen electrodes produce a flow of electrical current, the investigator can choose among the large number of hardware and software alternatives currently available for recording data. With the current generation of personal-computer-assisted data acquisition systems, the investigator can move directly from the raw data into a spreadsheet or database program and then into statistical analysis without ever having to touch a sheet of paper. Alternatively, data from the oxygen electrode can be recorded on a strip chart recorder and analyzed by hand.

Oxygen electrodes are, in the author's (J.J. Torres) opinion, the unequivocal best choice for determinations of metabolism in marine organisms from the size of small copepods to large fishes. However, they do not give the level of precision one can expect from the Winkler method in the hands of a well-trained marine chemist. Chemists participating in WOCE (World Ocean Circulation Experiment) are expected to show results reproducible to $\pm$ 0.010 ml l^{-1} (0.04 μmol kg^{-1}-sea water, about 0.2% of air saturated O_2 concentrations; Culberson 1991). In contrast, a good, well calibrated electrode is accurate to $\pm$ 1% of the oxygen reading at air saturation and should give highly repeatable numbers. Realizing from the outset that O_2 electrodes require a special patience in handling will go a long way toward successfully employing them. They are not as reliable or trouble-free as pH electrodes and should not be considered as such.

The most widely used oxygen electrode is the polarographic design of Clark (1956). For detailed information on the construction of Clark-type electrodes the compendium edited by Gnaiger and Forstner (1983) is a good source. A brief description of how Clark electrodes work is appropriate here, as it will aid the user in selecting the commercial electrode most appropriate for their application, or for the more adventurous, the diameter of platinum wire most appropriate for the electrodes they manufacture in their own laboratory.

The sensor tip of a Clark electrode is constructed of a platinum cathode and a silver–silver chloride anode (Figure 10.12). The anode and cathode are bathed in a 1 mol l^{-1} solution of KCl and the tip is sealed by an oxygen permeable polypropylene membrane about 25 μm in thickness, usually held in place with an O-ring. The polypropylene membrane effectively segregates the electrode tip from the outside medium; the pores in the membrane allow gases into the sensor tip but prevent interference from the larger

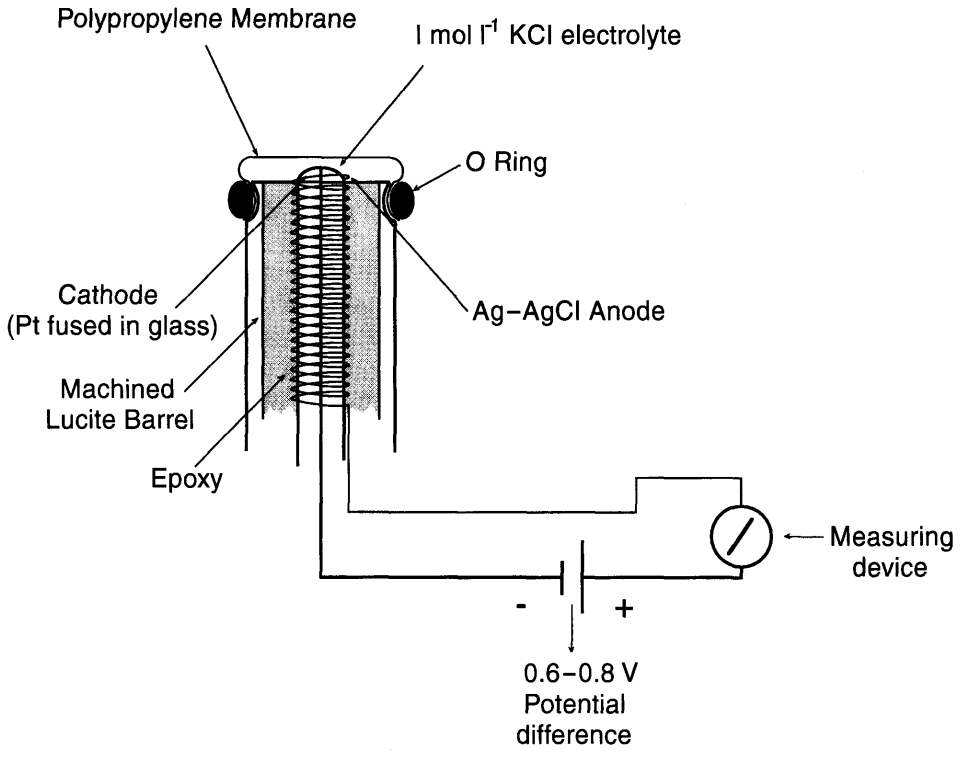

Fig. 10.12 A Clark oxygen electrode showing its major components. Platinum is fused in glass to make the cathode and is exposed only at the tip of the electrode. The silver–silver chloride anode and the cathode are bathed in an electrolyte composed of 1 mol l^{-1} KCl and sealed to the aqueous environment with a polypropylene membrane. See text.

hydrated ions of the salts in solution. Oxygen diffuses through the polypropylene membrane and is reduced at the platinum cathode, a current flow is induced by a five-step reaction that occurs continuously at the electrode tip (Figure 10.13):

At the Pt cathode (negative pole)	(1) $O_2 + 4e^- \rightarrow 2O^=$
Within the 1 mol KCl electrolyte	(2) $2O^= + 2H^+ \rightarrow 2OH^-$
	(3) $2OH^- + 2KCl \rightarrow 2KOH + 2Cl^-$
At the Ag–AgCl anode (positive pole)	(4) $2Cl^- \rightarrow 2Cl + 2e^-$
	(5) $Ag + Cl \rightarrow AgCl$

The oxygen ions created at the cathode tip combine with available hydronium ions in the electrolyte to form hydroxyl radicals. These in turn combine with the KCl in the electrolyte to release chloride ions. Chloride ions give up their electrons at the anode, yielding a current flow and an AgCl precipitate on the Ag–AgCl anode.

The reaction occurring at the electrode tip is interesting, easily understood, and underscores two important properties of the Clark electrode. First, the electrical current produced by the electrode is directly proportional to the partial pressure of oxygen within the medium. Second, by its very nature, the oxygen electrode itself consumes

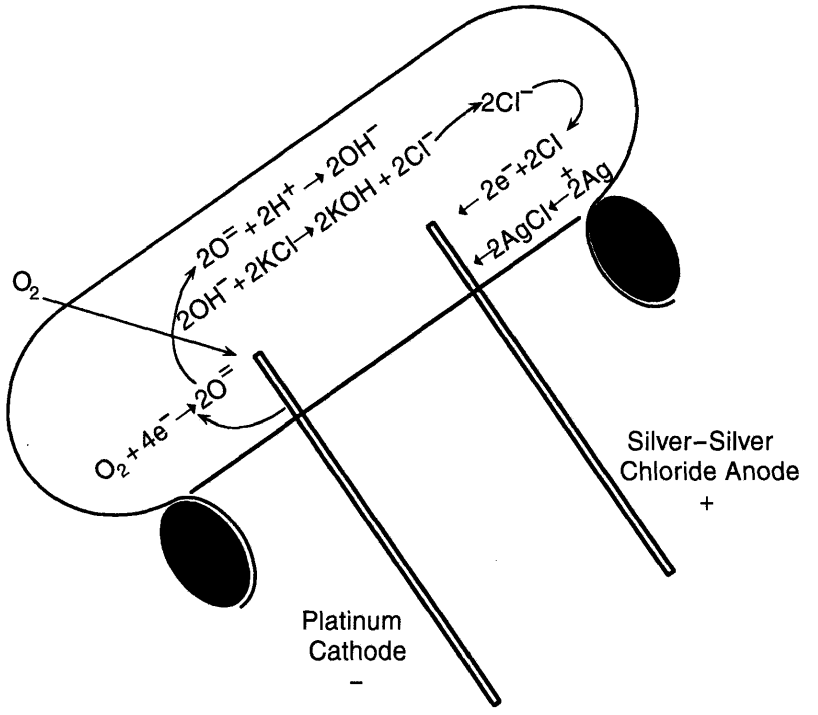

Fig. 10.13 Electrolyte dissociation at the tip of a Clark electrode. Oxygen diffuses through the polypropylene membrane and is reduced at the cathode, forming the oxygen ion. This initiates a series of reactions within the electrolyte that produces an electrical current in direct proportion to the oxygen in solution. See text.

oxygen. This second property of oxygen electrodes is important in their employment as a research tool and bears further examination.

At any interface of a fluid and a surface, such as that at the surface of the electrode membrane in sea water, a stagnant boundary layer forms due to the physics of fluid flow. If simple diffusion of oxygen through the boundary layer and membrane is not adequate to meet the oxygen demands of the electrode, the boundary layer will become depleted in oxygen. The electrode will then need to be stirred to minimize the thickness of the boundary layer and to facilitate diffusion of oxygen into the electrode tip. As we can discern from Figures 10.12 and 10.13 and the five-step reaction given above, the oxygen consumed by the cathode is directly proportional to its size, i.e. the diameter of the wire that is exposed at the tip. If the cathode is small enough, diffusion alone will provide sufficient oxygen to the electrode to obviate the need for stirring. Ideally, the cathode diameter should not be greater than 15 μm to eliminate the need for stirring.

A disadvantage of oxygen electrodes this small is the very low current output which requires substantial amplification. The need for amplification can make the electrodes more susceptible to electrical noise if they are not used with a well designed circuit. Another way of addressing the problem of stirring is to pulse the polarizing voltage of the electrode (Langdon 1984). To allow the electrolytic dissociation to proceed at the electrode tip as described above, a potential difference of 0.6 to 0.8 V must be maintained between the anode and the cathode of the electrode (Figure 10.12). If the polarizing voltage is turned off, the reaction at the tip will cease and the boundary layer at the tip of

the electrode will no longer be depleted in oxygen by the consumption of the electrode itself. In this manner, an electrode with a larger cathode can be used without the need for stirring. There is no reading taken during the time the electrode is off between pulses, making this design a little reminiscent of sequential sampling using the Winkler technique. Nevertheless, the electrodes employing this circuit design are widely used and have a good reputation for accuracy.

CHAMBERS

Apart from three basic requirements, the design of chambers for sealed jar respirometry is limited only by the needs and creativity of the investigator. The three basic requirements are, first, that the chamber be able to accept the electrode tip; second, that the chamber–electrode combination are sealed to the atmosphere; and third, that there is some means for rigorously controlling temperature within the chamber. Two useful chamber designs are shown in Figures 10.14 and 10.15 (cf. Quetin and Mickel 1983). In Figure 10.14 the tip of a syringe is cut off and the electrode itself forms an opposing plunger to the syringe plunger. In Figure 10.12, a water-jacketed lucite chamber has a chimney that mates with an O-ring on the barrel of the electrode. The syringe barrel chamber can be submerged within a water bath for temperature control while the water-jacketed chamber is connected to a circulating refrigerated water bath.

The design shown in Figure 10.14 has been successfully employed for respiratory determinations on individual copepods and fish larvae as small as 2 mm using unstirred electrodes. The design in Figure 10.15 is more suitable for larger species such as euphausiids and amphipods and can be used with stirred or unstirred electrodes. The cut-off for the minimum size of animal that is amenable to being an experimental subject

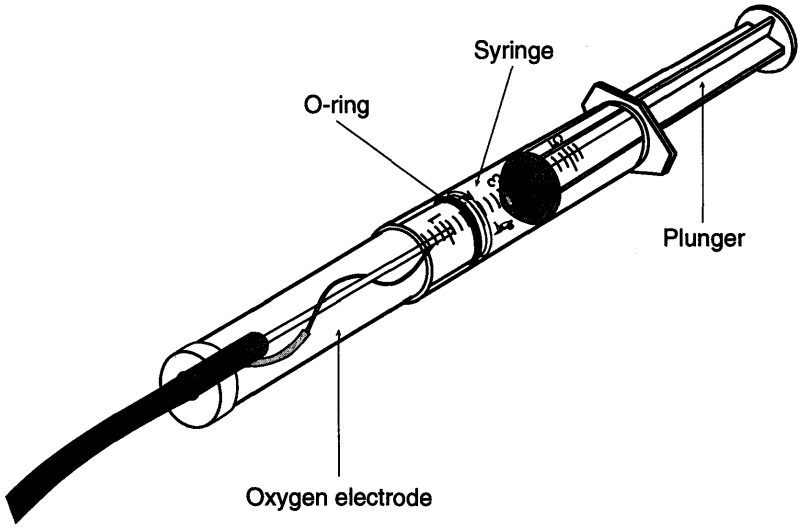

Fig. 10.14 A microrespirometer using an oxygen electrode and a cut syringe. The same O-ring that seats the electrode membrane seals the respirometer to the atmosphere. The assembled syringe–electrode combination as shown is placed in a water bath for temperature control. Alternatively, one can use the same principle with a luer-lock valve on the end of the syringe and using the electrode in place of the syringe plunger. The valve obviates the need for cutting the syringe.

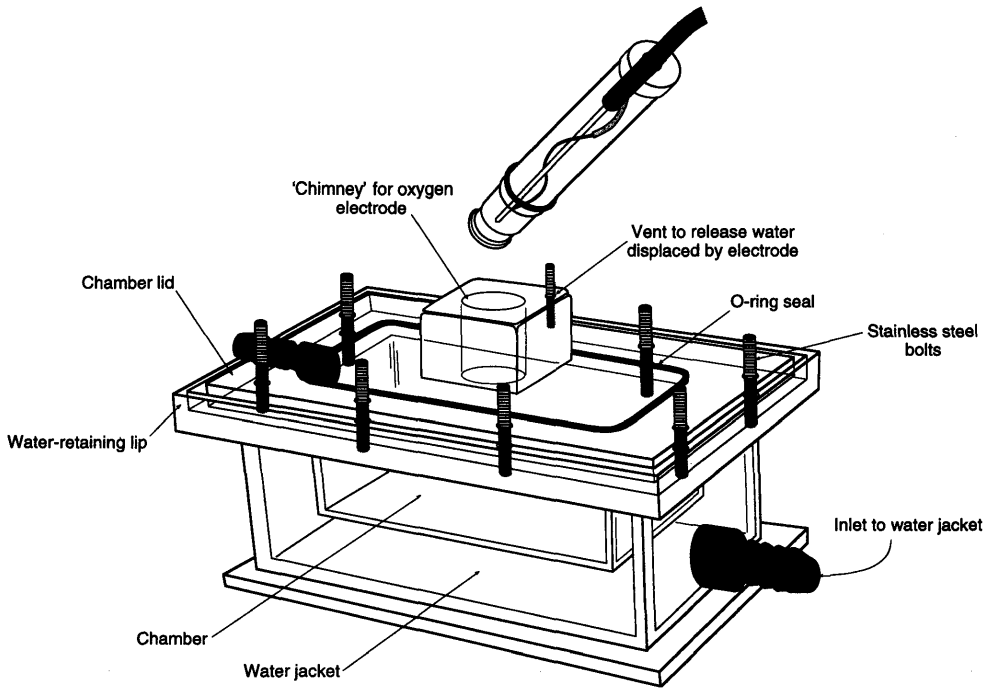

Fig. 10.15 A water-jacketed chamber for measuring oxygen consumption in larger zooplankton. The O-ring midway up the oxygen electrode makes a gas-tight seal on the walls of the chimney. A water-retaining lip allows the chamber to be overfilled to aid in eliminating bubbles. The chamber lid seats on an O-ring seal and is held down with wing nuts. The entire chamber is manufactured from lucite. Design by Joe Donnelly.

with stirred electrodes must depend on the good judgment of the investigator. Clearly, if an animal is swimming hard to maintain a position within the respiratory chamber against the current created by the stir bar, stirred electrodes are not appropriate. In no case is it appropriate to use stirred electrodes with gelatinous species.

Most investigators will be using electrodes available from commercial sources. Many come with choices of chamber types that are designed to mate with their electrode; some are very low volume systems that are suitable for use with zooplankton. If the investigator has just spent a considerable sum of money on an electrode system, he or she will be loathe to put their electrode in a lathe to modify it. In those cases the creativity and machine shop work will have to be restricted to the respiratory chamber. The investigator should, where possible, opt for flexibility in their chosen system.

PITFALLS

Temperature is a critical factor in respiratory determinations with the oxygen electrode, not only because of the effect of temperature on the metabolism of ectotherms but because oxygen electrodes are very sensitive to temperature. In fact, electrodes are more sensitive to temperature than they are to oxygen because the electrochemical dissociation occurring at the electrode tip is governed by the same Q_{10} rule as any biochemical reaction. Thus, while the solubility of oxygen at 10 °C is 123% of that at 20 °C, the current

flow generated at the electrode tip at 10 °C will be about 50% and so will the electrode output. Some commercially available electrodes are available with temperature compensation but keeping experimental and calibration temperatures identical is the simplest and best procedure.

When using the small volumes typical of experiments with individual zooplankton, bubbles can be a large potential problem in respiratory determinations. Oxygen is about 30 times more concentrated in air than in sea water; a very small volume of air can perturb the accuracy of a respiratory determination made in, for example, 2 ml of sea water. The investigator must be meticulous in removing all bubbles from the respiratory chamber. The best way to prevent bubbles is to avoid introducing them into the chamber to begin with. Siphoning water, using a large syringe, or pouring gently into your respiratory chamber will prevent introduction of bubbles. Once bubbles are in, microbubbles can be chased with larger bubbles or brushed out with small artist's brushes. Do not introduce cold water into a respiratory chamber and allow it to warm up. Bubbles will form as the solution degasses due to the reduced solubility of gases at warmer temperatures. Allow the water to equilibrate to experimental temperature first.

EXPERIMENTAL PROTOCOL

Electrodes are best calibrated immediately prior to, and directly after, experiments. In most cases, a high calibration is obtained by gentle aeration of the electrode in a calibration bath at experimental temperature or by using a combination of stirring and aeration. A low calibration is obtained by using a more violent bubbling with nitrogen gas in the calibration bath to drive off the oxygen in solution. Sodium sulphite is sometimes used to obtain a low calibration, but there is potential for poisoning a platinum cathode (see the five-step reaction given above) and it should be used with caution. Nitrogen gas, though inconvenient, does nothing but purge the oxygen from solution. Electrodes should ideally come to within 3% of electrical zero at the low end. Electrodes should be stable at the low and high ends and there should be little or no difference in high and low calibrations before and after the run. Differences of greater than 5% in the pre- and post-run high calibrations constitute grounds for rejection of a run. Linearity is assumed between the low and high points on the calibration.

The sea water to be used as a respiratory medium should be well filtered to remove microorganisms. The author (J.J. Torres) uses membrane filters (0.45 μm pore size) and has had little problem with control rates. Experimental subjects should be transferred to filtered water at experimental temperature prior to introduction into the respirometer as a rinsing step. After a satisfactory calibration has been obtained, the experimental subject is introduced to a bubble-free respiratory chamber and, taking extreme care not to introduce bubbles or disturb the subject more than absolutely necessary, the chamber is sealed, the electrode inserted, and the run is allowed to begin. It is best if visual stimulation is kept to a minimum by shading or isolating the animal in some way. If stirring is to be used, insuring that the magnetic stirring motor is fully warmed up prior to the run will prevent the magnetic stirrer speed-up that can result in homogenization of your experimental animal. It is best to avoid stirrer–hotplate combinations because both malfunctions in the hotplate and oversights due to fatigue can result in an unintended bouillabaisse instead of an experiment.

Control runs are used mainly to check for contributions by microorganisms to the total measured oxygen consumption rate. Since most microorganisms will be introduced by the experimental subject, it is best to take control rates at the termination of a run by removing the subject, replacing its volume with fresh filtered sea water, and

re-starting the run. Keeping respiratory chambers clean and using only filtered sea water for electrode calibrations and respiratory runs will go a long way to preventing errors when measuring control rates. Other uses for control runs are to check for electrode drift and oxygen consumption by the respiratory chamber or respiratory medium. In the latter two instances runs would be set up as normal, but without an animal.

Antibiotics are sometimes added to the filtered sea water employed as a respiratory medium to prevent microbial growth in the respiratory chamber. Those most commonly used are penicillin, streptomycin, and neomycin at concentrations of 25 to 50 mg l^{-1}. The authors' (J.J. Torres) experience has been that microbial growth is a negligible problem within the time frame of a typical run (8 to 12 h) when working with filtered sea water at temperatures less than 15 °C. Microbial growth needs to be more carefully monitored at higher temperatures and antibiotics can help to control this. Runs greater than 12 h in duration need to be checked carefully for microbial contributions at any temperature but especially at temperatures above 15 °C.

The recording apparatus should be consulted frequently. Notes on animal activity and condition should be made at intervals as unobtrusively as possible. Low oxygen becomes stressful to most pelagic species at about 25% of air saturation (e.g. Donnelly and Torres 1988; Torres *et al.* 1994); they can no longer maintain a constant oxygen consumption rate below this level. It is best if the chamber volume allows the animals to remove 5% to 10% of the oxygen in the chamber each hour.

CALCULATIONS AND TREATMENT OF DATA

Calculations of metabolism with data from oxygen electrodes use the same principles regardless of the recording system. Two leaps of faith are required when equating the electrical output of oxygen electrodes to quantities of dissolved oxygen. The first is that the electrical current produced by the electrode varies directly and linearly with the dissolved oxygen in the range from zero to air saturation. The second is that the high calibration point is equivalent to the oxygen solubility at the temperature and salinity in the calibration bath.

The assumption of linearity can be checked by testing the output of the electrode at air saturation as you would during a normal calibration and then at oxygen saturation by using a tank of pure oxygen. This will then give you the output at 0%, 21% and 100% O_2, enough points to check for linearity of response. Alternatively, you can use the Winkler method or a gas chromatograph to check at various points between zero and air saturation. The author (J.J. Torres) has never noted a problem with linearity.

The assumption that the high calibration point of the electrode is equivalent to the oxygen solubility at the calibration temperature and salinity is necessary to convert the electrical output of the electrode to the units of dissolved oxygen that are of use to us. Values for oxygen solubilities over a wide range of temperatures and salinities are available from the exacting research of marine chemists. The most widely accepted numbers for oxygen solubility are those from the equation of Weiss (1970), who, using the data of Carpenter (1966) and Murray and Riley (1969), described the relation between temperature, salinity and oxygen solubility. Weiss's equation, as reported in Kester (1975; equation 8.11) is:

$$\ln c^* = A_1 + A_2(100/T) + A_3 \ln(T/100) + A_4(T/100) + S‰[B_1 + B_2(T/100) + B_3(T/100)^2] \quad (10.16)$$

for c^* = O_2 solubility in μmol kg^{-1} based on the data of Carpenter (1966) then: A_1 = −173.9894, A_2 = 255.5907, A_3 = 146.4813, A_4 = −22.2040, B_1 = −0.037 362, B_2 = 0.016 504, and B_3 = −0.002 0564, and for c^* = O_2 solubility in $cm^3\ l^{-1}$ based on the data of Murray and Riley (1969) then: A_1 = −173.4292, A_2 = 249.6339, A_3 = 143.3483, A_4 = −21.8492, B_1 = −0.033 096, B_2 = 0.014 259, and B_3 = −0.001 7000.

Values assume an atmosphere of 20.94% O_2 at 760 mmHg total pressure and at 100% relative humidity. As an alternative to using the equations above, one can look up the tabulated values in either of the original papers and use those. The differences in solubilities between the original papers and Weiss's improved numbers are small enough ($\leq 0.03\ cm^3\ dm^{-3}$) for our purposes to make the error an acceptable one. Reading any of the original papers on oxygen solubility and especially Kester's treatment will familiarize the investigator with units, accuracy, and precision in determining values for dissolved gases.

The electrode output at the low and high calibration points is assumed to be 0% and 100% of air saturation respectively. The O_2 solubility determined from Weiss's equations or from the original tabulated data at the experimental temperature and salinity yields the total dissolved oxygen at air saturation. The investigator can then compute the oxygen consumption from the percent change in total electrode output with time. For example:

- electrode output at high cal is 100 mV which equals O_2 solubility at air saturation: 7.10 ml l^{-1}
- electrode output at low cal is 0 mV
- total volume of respirometer: 5 ml
- total oxygen in system: 35.5 μl
- wet mass of experimental subject: 10 mg

After 1 h the electrode reads 90 mV, 10% of oxygen in the system has been removed. Oxygen removed from system is 3.55 μl, oxygen consumption rate is 0.355 μl O_2 mg wet mass $^{-1}$ h^{-1}.

10.7.3 Enzymatic method – electron transfer system (S. Hernández-León)

The Electron Transfer System (ETS) is the pathway responsible for transfer of electrons to oxygen, the final electron acceptor. As a consequence, its activity is responsible for oxygen consumption by both the cell and organism, and can be used as an index, or biochemical proxy for zooplankton respiration in the sea (section 10.4.1). ETS may be characterized as a multi-enzyme, multi-substrate system, and its activity is determined in substrate saturating conditions, i.e. at the maximal rate (V_{max}). The substrates are nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), and succinate, which saturate microsomal and mitochondrial ETS. The tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) is used as an artificial electron acceptor to measure the electron transmission rate: a mole of oxygen consumed will be equivalent to two moles of INT reduction. The slightly yellow INT is reduced to the pink colored formazan and the intensity of the color is read with a spectrophotometer.

The enzymes involved in the electron transfer system are NADH dehydrogenase (EC 1.6.99.3), NADPH dehydrogenase, (EC 1.6.99.6), NADPH-cytochrome reductase (EC 1.6.2.4) and succinate dehydrogenase (EC 1.3.99.1). The activity of these enzymes are recorded in the same assay and the ETS activity is not necessarily the addition of the

activities of the different enzymes of an enzyme complex (Savenkoff *et al.* 1995; Gómez *et al.* 1996). NADH dehydrogenase is the most active dehydrogenase in zooplankton material.

Since the work by Packard (1971) the assay method has been modified by the addition of Triton X-100 (Owens and King 1975), and by changes in pH of the buffers (Kenner and Ahmed 1975). The assay method described here is essentially the same as that of Kenner and Ahmed (1975) and Packard and Williams (1981), modified slightly by Gómez *et al.* (1996) for zooplankton.

REAGENTS

1) Homogenization buffer 0.05 mol phosphate buffer, pH 8.0, containing 6.72 g Na_2HPO_4, 0.362 g KH_2PO_4, 1.5 g polyvinyl pyrrolidone (PVP), 18.5 mg $MgSO_4.7H_2O$ and 2 ml Triton X-100, in 1 l of deionized-distilled water (DDW). Store at −20 °C.
2) Daily homogenization buffer Add, immediately before use, an appropriate amount of 0.1 mol NaCN (e.g. 0.735 g NaCN in 150 ml of DDW) to obtain a final concentration of 2 mmol NaCN (e.g. 1 ml NaCN solution in 49 ml of the homogenization buffer).
3) Substrate buffer Prepare a solution of 0.05 mol phosphate buffer, pH 8.0, containing 6.72 g Na_2HPO_4, 0.362 g KH_2PO_4, 36 g disodium succinate hexahydrate and 2 ml Triton X-100. Store at −20 °C.
4) Daily substrate solution Just before the assay, dissolve 15 mg NADH and 5 mg NADPH in 24 ml of the substrate buffer.
5) Blank substrate buffer Prepare the substrate buffer (3) without substrates as a blank. Store at −20 °C.
6) INT solution Prepare a solution of the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrasolium chloride (INT) in DDW up to a concentration of 4 mmol (e.g. 1 g INT in 500 ml DDW). Filter the solution and store at −20 °C in the dark.
7) Quench solution 50% formalin (36%) plus 50% 1 mol H_3PO_4, pH 2.5. Store under refrigeration (0–4 °C).

Bacterial contamination should be kept to the absolute minimum when mixing up solutions. To achieve this, working in very clean conditions is essential, and solutions should not be kept for long at room temperature.

SAMPLE PREPARATION AND PRESERVATION

Sorted live zooplankton is ready to use for the assay after removing excess water with blotting paper. For mixed zooplankton, samples in the net cod-end are poured onto a 100 μm mesh sieve and washed with filtered sea water. The mesh sieve can then be put over blotting paper to remove excess water. If samples are too large to apply this procedure, they may be gently vacuum filtered. Samples are scraped off the filters using a spatula for the assay. Fresh samples are best, but freezing samples may be inevitable in the experiments at sea.

Ahmed *et al.* (1976) observed that although a cell-free extract of the copepod *Calanus finmarchicus* frozen at −2 °C lost considerable ETS activity in 24 h, no measurable loss was found when intact specimens were stored at −20 °C or below for at least one week. No significant loss of ETS activity during storage for 34 days at −20 °C has been reported for two fish species (Ikeda 1989b). On the other hand Båmstedt (1980) found

that frozen intact specimens of *Acartia tonsa* lost 50% of their ETS activity within 30 h at −20 °C and virtually no activity was detected after 72 h.

Recently, Gómez *et al.* (1996) observed no significant loss in ETS activity on zooplankton samples preserved in liquid nitrogen (−196 °C) for at least 2 months. Therefore, preservation of zooplankton samples in liquid nitrogen is recommended for the ETS assay, whenever the immediate assay is not practicable.

ASSAY PROCEDURE

1) The zooplankton sample is homogenized with or without GF/C filters (Notes for the assay procedure and calculations, section 10.7.3, part a) in a teflon-glass tissue grinder (Notes for the assay procedure and calculations, 10.7.3, part b) for 2 min at 0 to 4 °C in a known volume of homogenization buffer (approximately 10–20% (v/v) homogenate) (Notes for the assay procedure and calculations, section 10.7.3 part c)
2) The homogenate is then poured into a chilled graduated cylinder in order to record the volume. The difference between this volume and the volume of buffer used for homogenization is the wet volume of the sample.
3) Centrifuge (Notes for the assay procedure and calculations, section 10.7.3 part d) for 10 min at 1000 *g* at 0 to 4 °C. Because the method is very sensitive the supernatant must be diluted in homogenization buffer. A 3 to 61 × dilution is often used depending on the concentration of sample in the homogenate.
4) Take a 1-ml aliquot of diluted supernatant, mix with 3 ml of the daily substrate solution and 1 ml of INT solution, and incubate for 20 min at the *in situ* temperature in the dark. The color of the reaction mixture changes during the incubation period to pink due to the reduction of the INT to formazan. Red color is indicative of excess sample. Concurrently, another 1 ml of the diluted supernatant is incubated in the blank substrate buffer (substrate blank). Get another blank to account for the reaction between substrate and INT (reagent blank). Take 1 ml of daily homogenization buffer and incubate using the same procedure as with the sample.
5) Stop the reaction with 1 ml of the quench solution.
6) Read absorbance with a spectrophotometer at 490 nm and at 750 nm as the turbidity base line. The increase in absorbance can be monitored continuously by using a temperature-controlled cuvette placed in a spectrophotometer. New technologies such as microplate readers and robotic samplers can also be used to save time when a large number of samples has to be processed.

In order to obtain specific rates, measure protein contents of the homogenized samples obtained at step 3, by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard, or modified Lowry *et al.*'s method by Peterson (1977, 1983) for samples with very low protein content.

Please note that Savqenkoff *et al.* (1995) suggested the use of a yeast (*Saccharomyces cerevisiae*) as a reference to standardize ETS assays. The yeast is available in dried form which can be weighed and stored. The procedure consists of weighing five samples of the yeast from 1 to 5 mg with an ultramicrobalance using its large grains. Follow the steps given in the assay procedure but homogenize together with GF/C filters (diameter: 25 mm) in order to disrupt mitochondria walls. Plot weights against ETS activity. ETS activity data from dissimilar assay procedures by different workers could be compared with the reference material.

CALCULATIONS

ETS activity is calculated as:

$$ETS = 60 \times H \times AS \times COD/(\mathrm{INT} \times T \times L \times f), \quad (10.17)$$

$$COD = [(AOD \times AS) - (BOD \times BS) - (FOD \times FS)]/AS, \quad (10.18)$$

where H is the homogenization volume, AS is the assay volume, BS is the blank volume, FS is the reagent blank volume, AOD is the difference between the spectrophotometer readings at 490 and 750 nm of the assay (BOD and FOD are the same differences for substrate blank and reagent blank, respectively), T is the incubation time (minutes), f is the volume of homogenate used in the assay (ml), L is the path length of the spectrophotometer cuvette cell used (usually 1 cm), 60 is the factor to convert minutes to hours, and INT is the standardization factor (see below) which converts the absorbance of the produced INT-formazan. The ETS activity is given in $\mu l\ O_2 h^{-1}$ but could be expressed in electron equivalents (μEq e^-) by dividing the ETS activity by 5.6 (1 mol of O_2 = 4 Eq e^- and $ETS \times 4/22.4 = ETS/5.6$).

ETS assays are normally run at one temperature during a set of experiments, or during a cruise. The experimental temperature may not be the *in situ* temperature. ETS activity obtained at a given incubation temperature may be converted to the activity at *in situ* temperature using the following equation:

$$ETS_{in\ situ} = ETS_{incu} \times \exp[E_a(1/T_{incu} - 1/T_{in\ situ})/R]$$

The value of the Arrhenius activation energy (E_a) is about 15 kcal mol^{-1} in *Calanus finmarchicus* and in the range of 13 to 16 kcal mol^{-1} for a variety of other plankton. The symbol R is the gas constant (1.987×10^{-3} kcal mol^{-1}) and T is the absolute temperature (Packard *et al.* 1975).

Convert ETS activity to respiratory oxygen consumption rate using the correlation between respiration rate and ETS activity (R/ETS ratio). The R/ETS ratio is theoretically around 0.5. Problems associated with the R/ETS ratio are detailed in ETS activity, section 10.4.1.

INT STANDARDIZATION

Because of different qualities (or different trade marks) of INT, it is highly recommended that you standardize it using the procedure given below.

REAGENTS AND SOLUTIONS

1) 0.1 mol phosphate buffer Add 13.6 g KH_2PO_4 to 1 l of DDW. Add 14.2 g Na_2HPO_4 to 1 l of DDW. Mix 808 ml of the Na_2HPO_4 solution with 192 ml of the KH_2PO_4 solution. Add 10 ml Triton X-100 to the mixed solution. Store at −20 °C.
2) INT color reagent Add 8 mg phenazine methosulphate (PMS) and 0.4 ml Triton X-100 to 16 ml of the INT solution to be standardized.
3) 0.05 mol phthalate buffer Dissolve 2.55 g phthalic acid in 172 ml of DDW. Add 51 ml of 0.1 *N* HCl and 22 ml Triton X-100. Adjust to pH 3.0 if necessary. Dilute to 250 ml.

Standardization procedure

1) Prepare a solution of 0.043 μmol ml^{-1} of NADH in 0.1 mol phosphate buffer, pH 7.4 (e.g. 2 mg NADH in 60 ml of 0.1 mol phosphate buffer).
2) Prepare a series of dilutions to obtain different concentrations between 0 and 0.258 μEq e^- in a total volume of 3.0 ml.

3) Add 2.0 ml of INT color reagent to each tube and incubate in darkness for exactly 1 min at room temperature. Stop the reaction with 1.0 ml of 0.05 mol phthalate buffer. Total volume of the assay is 6.0 ml. Read absorbance at 490 and 750 nm with a spectrophotometer.

The factor used in the standardization is calculated converting the μmol NADH ml^{-1} to μEq e^- (multiply μmol ml^{-1} by 2). Plot absorbance against the μEq e^- and obtain a slope from a least square linear regression. Then, multiply the slope by 1.24 obtaining the value to convert the absorbance of the INT-formazan produced in the calculation of ETS. This value should be around 1.42 because the INT-formazan has a molar extinction coefficient (A_{490}) in 0.133% Triton X-100 solution of 15.9×10^3 mol^{-1} cm^{-1}. INT requires two electrons to be reduced to formazan and oxygen requires four electrons to be reduced to water. Therefore, the extinction coefficient of INT-formazan is equivalent to a 0.5 molar solution or 11.2 l O_2 l^{-1}. Because the solvent volume is taken into account in the ETS assay, the equivalent absorptivity of 1 μl O_2 per ml of solvent will be $15.9 \times 10^3/(11.2 \times 10^3) = 1.42$.

NOTES FOR THE ASSAY PROCEDURE AND CALCULATIONS

a) The use of GF/C filters is to facilitate disruption of mitochondria walls. However, Gómez *et al.* (1996) found no significant difference between ETS activities of mixed zooplankton samples processed with and without GF/C filters, and suggested that chitin exoskeleton of crustacean zooplankton had the same function as GF/C filters for crustacean zooplankton samples or mixed zooplankton samples which include crustaceans.

b) A Polytron ultrarapid homogenizer may be used instead, as Gómez *et al.* (1996) noted no significant differences between the resultant ETS activities of samples processed with teflon-glass grinder and the Polytron ultrarapid homogenizer. Sonication may also be an alternative, but the author (S. Hernández-León) has not tested this yet.

c) Crude homogenates of zooplankton can be maintained in a water-ice bath (0–4 °C) for up to 90 min without any significant loss in ETS activity (Båmstedt 1980; Gómez *et al.* 1996) thus allowing preparation of several samples before the start of the assay.

d) While Gómez *et al.* (1996) observed no significant differences in ETS activities of samples centrifuged before and after the incubation, the procedure given here (centrifuge before the incubation) is recommended to avoid an increment of turbidity in the assay.

10.7.4 Enzymatic method – lactate dehydrogenase and citrate synthase (J.J. Torres and S.P. Geiger)

Lactate dehydrogenase (LDH) and citrate synthase (CS) catalyze key reactions of anaerobic and aerobic intermediary metabolism respectively. LDH is the terminal enzyme of anaerobic glycolysis as the pathway is conventionally presented; it catalyzes the conversion of pyruvate to lactate and is one of the three rate-limiting steps in the pathway. CS catalyzes the condensation reaction between acetyl-CoA and oxaloacetate to form citrate, which is the first and rate-limiting step in the Krebs cycle. The rationale behind using LDH and CS as indicators of metabolism is, first, that the activity of each enzyme is indicative of the metabolic poise (cf. Hochachka and Somero 1984), or activity, of its respective pathway, and second, that the activity of the pathway relates

directly to the metabolism and therefore the overall physiological condition of the whole organism (see Enzymes of intermediary metabolism in section 10.4.2).

LDH is best applied as a physiological indicator in ichthyoplankton (Clarke *et al.* 1992; Brightman 1993). The data available suggest that LDH activities in ichthyoplankton correlate well with growth and physiological condition, and are further supported by years of research on the biochemical physiology of vertebrates (Hochachka and Somero 1984). Less is known about the function of LDH in crustaceans and other zooplankton. It is present in detectable quantities (e.g. Thuesen and Childress 1993a, 1993b, 1994; Geiger *et al.* 1996; Thuesen *et al.* in press) but its function in invertebrate metabolism is less well described and variability even between species of a general taxonomic group, e.g. calanoid copepods, can be very high. For example, results on LDH activities in Antarctic copepods (S.P. Geiger and J.J. Torres unpublished data) using the techniques described in Lactate dehydrogenase, section 10.7.4 suggest low, but detectable, levels of LDH, whereas results described in Thuesen *et al.* (in press) suggest very high activities in deep-living copepods of the California Current. Invertebrates exhibit considerably more creativity with their glycolytic endpoints than do vertebrates (Hochachka and Somero 1984). Thus, the meaning of LDH activity should be evaluated for invertebrate preparations on a case-by-case basis.

CS, while it is a slightly more difficult assay, seems to work equally well on both vertebrate and invertebrate subjects and is especially useful in copepods. We recommend it as a first choice for assaying condition in copepods and LDH as a first choice in ichthyoplankton.

TISSUE PREPARATION AND GENERAL PROCEDURES

Enzyme activities are most stable when tissue is frozen initially in liquid nitrogen, and then stored at −80 °C. No loss in enzyme activity occurs for at least several months and for most enzymes, storage at −80 °C allows nearly indefinite storage. Both LDH and CS are fairly stable enzymes and if the ideal situation cannot be achieved, then investigators should do their best to approach them and be wary of deterioration. If a choice must be made, the initial (liquid nitrogen) freezing step is the most critical one and the samples can usually then be stored in a conventional (−20 °C) deep-freeze with little loss of activity for a few months. A word of caution: tissues and enzymes do vary in their sensitivity to storage, so these procedures should be viewed as general guidelines and not gospel. Gelatinous organisms and small copepods are particularly sensitive to desiccation with long term storage. It is best to determine empirically the sensitivity of your own preparations to storage. Once homogenized, samples should be used within several hours and not be refrozen.

Homogenize tissue in ice-cold 50 mmol imidazole buffer (pH 7.2 at 20 °C) using either a sonicator or conventional homogenizer. The dilution factor to be used varies with the tissue and its inherent enzyme activity. It is imperative that the dilution factor be known precisely because it is an important element in calculating enzyme activity and expressing it in standard units (see Calculations, section 10.7.4). For tissues with low activity, a dilution factor of 1 to 4 (1 g tissue to 4 ml buffer) is useful, for tissues with more enzyme a 1 to 24 dilution is more appropriate. Large tissue samples (e.g. *Euphausia superba* abdominal muscle) should be ground in chilled ground glass homogenizers, and then briefly sonicated. For small tissue samples (e.g. calanoid copepods, or young < 10 mm fish larvae) sonications usually provide adequate homogenization. Large tissue samples are best centrifuged at 2500 to 5000 *g* for 10 min to reduce particulate interference during the assay.

In some cases it is better to initially homogenize tissues in ice-cold distilled water. If your sample is too small or too delicate to obtain a reliable initial weight (e.g. a small copepod frozen in a cryovial) and you wish to normalize your activities to protein, you can add a known volume of distilled water to the vial and homogenize 'in place' with a sonicator. We have found no significant loss in enzyme activity when compared to homogenizing in buffer, and homogenizing in water allows you to perform multiple assays on single samples. Some buffers, notably Hepes buffer, interfere with the Lowry protein assay and with the Ethidium Bromide technique for determination of nucleic acids. An initial homogenization step in distilled water will allow you to obtain protein concentration in your homogenate, thereby giving you a standard for comparison with other investigators and with other values in your own data set.

Enzyme activity is very temperature-sensitive, a change of 1 °C in the cuvette will result in a 10% change in reaction rate. Thus, a spectrophotometer with thermally controlled cuvette holders (or a plate reader with thermal control) is imperative for determinations of enzyme activity. Cuvettes should be allowed to equilibrate to experimental temperature to ensure a stable, comparable reaction rate.

PITFALLS

It is *extremely* important to measure accurately the volume of homogenate that you add to the reaction cuvette. Use the smallest pipette available for adding homogenate and be certain to wipe off any small drops from the outside of the pipette before dangling it over the cuvette. It is often easiest to use the pipette to stir thoroughly the homogenate into the assay buffer, stirring is important. Properly maintained (calibrated!) automatic pipettes such as the Pipetman are fine for enzyme assays. Some old-timers (such as the senior author and his mentor) prefer to use Lang–Levy mouth pipettes to add the homogenate to the reaction cuvette. A gentle exhalation after dispensing homogenate into the cuvette produces a small flow of bubbles; this, when followed by hand stirring, mixes the homogenate in very thoroughly.

It is important to keep track of the daily order of assays as preparations may change in activity during the day. Protease activity may cause your homogenate to deteriorate with time, cocktails may evaporate, resulting in an increased concentration of reactants, and a host of other things can potentially affect your results. Keep good notes; if you observe changes you may be able to address them later. *Remember that enzyme assays using NADH should be kept shielded from light as much as possible. NADH is very photoreactive.* Wrapping all beakers in aluminum foil works well. Also try to keep the beakers covered, either with foil or parafilm, to prevent excessive evaporation.

Imidazole buffer changes pH at a rate of -0.02 units/°C^{-1}. This rate of change is very similar to that observed in animal tissue. All buffers have a characteristic range of change in pH with temperature. You must account for this change if you are mixing up buffers at room temperature and plan to run them at a different temperature. Be sure that you are using a pH electrode that is suitable for use with organic solutions.

The pHs chosen for LDH and CS in the protocols given below (in section 10.7.4 on Lactate dehydrogenase and Citrate synthase) are optimal, or very close to optimal, for the broad array of fishes and crustaceans, including copepods, that have been examined in our (J.J. Torres and S.P. Geiger) laboratory. CS is a mitochondrial enzyme and requires a slightly more alkaline milieu than LDH, a muscle enzyme, to more accurately mimic the conditions in the mitochondrial matrix. We recommend the pHs given below as an excellent place to start your enzyme studies, and if further optimization for your

experimental organism is warranted by the character of your study, a pH series can be run to fine tune the assay. pH is important, for example, the direction of the LDH assay can be reversed at too high a pH.

Other potential sources of error that are easily addressed are dilution effects in your homogenate, i.e. is your homogenate homogeneous? If you suspect problems, a test for linearity by using half and twice the homogenate concentration in successive activity determinations can be performed to test for potential problems in the dilute homogenates typical with zooplankton samples. If you obtain a non-linear result, you can adjust your homogenate volume until reproducibility is obtained.

The substrate concentrations given below are greatly in excess, which will provide you with V_{max} numbers for your homogenates. None the less, if you suspect substrate limitation in your enzyme, testing for linearity by halving and doubling substrate concentrations in the cocktail is a good way of verifying that substrates are not limiting.

LACTATE DEHYDROGENASE (PYRUVATE + NADH + H^+ ↔ LACTATE + NAD^+; ABSORBANCE AT 340 NM DECREASES AS NADH IS OXIDIZED TO NAD^+)

All solutions should be mixed up to achieve pH 7.2 at 20 °C. The final assay medium should contain the following:

- 80 mmol imidazole buffer
- 0.15 mmol NADH
- 5.0 mmol Na-pyruvate.

To make 50 ml of assay cocktail follow these procedures.

1) Make up a stock of 200 mmol imidazole buffer (Sigma No. I-0125): 3.4 g 250 ml^{-1} H_2O.
2) Make up a stock of 50 mmol imidazole buffer, 0.85 g 50 ml^{-1} H_2O. Refrigerated stock of 200 mmol and 50 mmol imidazol buffer can be stored for several weeks.
3) Make 3.25 mmol NADH fresh daily (Sigma No. N-8129), 0.0115 g 5 ml^{-1} 50 mmol imidazole.
4) Make up 100 mmol Na-pyruvate fresh daily (Sigma No. P-2256), 0.110 g 10 ml^{-1} of 50 mmol imidazole (Note: NADH and Na-pyruvate need not be mixed to exactly 5 or 10 ml. Simply weigh out a small amount, then add enough buffer to reach the desired concentration. This will be 0.435 ml buffer mg^{-1} NADH, and 0.091 ml buffer mg^{-1} Na-pyruvate).
5) To make the assay cocktail (make it fresh daily) add 20 ml of imidazole buffer (solution 1), 2.5 ml each of NADH (solution 3) and Na-pyruvate (solution 4), and 25 ml of H_2O.

Add 10 to 20 μl homogenate to 2 ml of the assay cocktail. Observe the change in absorbance at 340 nm for 30–90 s. The slope will decrease rapidly after an initial linear interval. Adjust the homogenate volume to allow the slope of the reaction during the initial linear phase to be easily determined.

CITRATE SYNTHASE (ACETYL-COA + $OXALOACETATE^{2-}$ + H_2O ↔ $CITRATE^{3-}$ + COASH + H^+)(−SH DISSOCIATES FROM COASH AND REACTS WITH DTNB)

All solutions should be mixed up to achieve pH 8.0 at 20 °C. The final assay medium should contain the following:

- 50 mmol imidazole buffer
- 0.1 mmol acetyl-Coenzyme-A (Boehringer-Manheim No. 10197, preferred over Sigma Acetyl CoA)
- 0.2 mmol DTNB (5,5* Dithio-bis(2-Nitrobenzoic acid)
- 0.5 mmol oxaloacetic acid.

To make 50 ml of assay cocktail follow these procedures.

1) Make up a stock of 50 mmol imidazole buffer (Sigma No. I-0125, 0.85 g 50 ml H_2O). This stock can be refrigerated for several weeks.
2) Make 4 mmol DTNB fresh daily (Sigma No. D-8130, 16 mg 10 ml^{-1} 50 mmol imidazole).
3) Make 40 mmol oxaloacetate fresh daily (Sigma No. 0-4126, 53 mg 10 ml^{-1} 50 mmol imidazole). Adjust pH to near 7.0 before bringing total volume to 10 ml (approximately eight drops of 2*N* NaOH or KOH).
4) To make the assay cocktail mix 47.5 ml of imidazole buffer (solution 1), 2.5 ml DTNB (solution 2), and 5 mg Acetyl-CoA (Acetyl-CoA is very expensive, roughly one US dollar per 2 ml assay, so do not make up more assay buffer than you need for that day. By reducing the assay to 1 ml cuvettes you can reduce this cost by 50% and also use less homogenate, allowing smaller tissue samples to be used. However, this may increase the noise in the assay slightly).

Add 10 to 50 μl homogenate to 2 ml assay cocktail to observe the background change in absorbance at 412 nm for 1 to 3 min. Once the slope has flattened out, add 25 μl oxaloacetate and observe the change in absorbance for an additional 2 to 5 min. This slope will often require a 30 to 60 s lag time after the addition of oxaloacetate before becoming linear.

CALCULATIONS

The rate of change of reactant to product during steady state conditions at the initial velocity of an enzyme assay is a measure of the activity of an enzyme. International units of enzyme activity (IU) are μmoles substrate converted to product per minute. IUs of enzyme activity are most often standardized to either the weight of tissue being assayed (e.g. IU per mg wet weight) or to protein concentration (IU per mg protein). If the enzyme is saturated with substrate, and not inhibited by product, then under constant conditions the rates of enzyme reaction reduces to the following:

$$\text{Rate} = k[E].[s] \tag{10.20}$$

where k is specific to most species and assay conditions, $[E]$ is the concentration of enzyme, and $[s]$ is the concentration of substrate. Remember that you are not measuring exact *in vivo* rates but only consistent *in vitro* rates.

The Beer–Lambert law describing the relation between the optical density of a solution and its concentration says:

$$A = e \times C \times L \tag{10.21}$$

where A is absorbance, e is the extinction coefficient of solute in l mol^{-1}-cm (also seen as 1 = 1000 cm^3, making the equation cm^2 mol^{-1}), for NADH, $e = 6.22 \times 10^6$ at 340 nm, and for DTNB, $e = 13.6 \times 10^6$ at 412 nm, C is the concentration of substance in mol l^{-1}, and L is length of light path in cm (generally 1 for the most common cuvette size).

Setting the equation above equal to concentration we get: $C = A/(e \times L)$. The enzyme activity is the change in concentration of substrate per unit time, which in turn is described by the change in absorbance per unit time. To express enzyme activity in μmole product converted per minute:

$$(\Delta A/\text{min}^{-1})/(e \times L) = (\Delta A\ \text{min}^{-1})/[(6.22 \times 10^6\ \text{cm}^2\ \text{mol}^{-1}) \times 1\ \text{cm}] = (\Delta\text{mol ml}^{-1})\ \text{min}^{-1} \quad (10.22)$$

The Δmol ml^{-1} min^{-1} can then be multiplied by the concentration of tissue in the homogenate to standardize to wet mass.

For example, you have added 10 μl of a 1:4 homogenate to 2 ml of reaction cocktail to determine LDH activity. You obtain a rate of change in absorbance of 0.026 absorbance units per min. Thus, $0.026/[(6.22 \times 10^6\ \text{cm}^2\ \text{mol}^{-1}) \times 1\ \text{cm}] = 4.2 \times 10^{-9}$ mol min^{-1} ml^{-1} $= 4.2 \times 10^{-3}$ μmol min^{-1} ml^{-1}. For 2 ml of cocktail, total activity is: 4.2×10^{-3} μmol min^{-1} ml^{-1} $\times$ 2 $= 8.4 \times 10^{-3}$ μmol min^{-1}. For a 1:4 dilution the concentration of tissue is 0.2 g 1000 μl^{-1}. You used 10 μl of the 1:4 homogenate, so to obtain the activity for 1 g of tissue, (1000 μl 0.2 g^{-1}) $\times$ 1/10 μl = 500, and $500 \times 8.4 \times 10^{-3}$ μmol min^{-1} $= 4.2$ μmol min^{-1} g^{-1} wet mass.

More simply, for LDH: (2 ml cocktail/6.22) $\times$ (ΔAbsorbance/min) = No. of μmoles substrate converted to product per minute. For CS simply substitute 13.6 (extinction coefficient) for 6.22.

FURTHER READING

Alp, P.R., Newsholme, E.A. and Zammit, V.A., 1976. Activities of citrate synthase and NAD$^+$ and NADP$^+$-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates. *Biochemical Journal*, **154**: 689–700.

Somero, G.N., 1981. pH–Temperature interactions on proteins: principles of optimal pH and buffer system design. *Marine Biology Letters*, **2**: 163–178.

Srere, P.A., 1969. Citrate synthase. *Methods in Enzymology*, **13**: 3–11.

Sugden, P.H. and Newsholme, E.A., 1975. Activities of citrate synthase, NAD$^+$-linked and NADP$^+$-linked isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in nervous tissues from vertebrates and invertebrates. *Biochemical Journal*, **150**: 105–111.

Walsh, P.J., Bedolla, C. and Mommsen, T.P., 1989. Scaling and sex related differences in toadfish (*Opsanus beta*) sonic muscle enzyme activities. *Bulletin of Marine Science*, **45**: 68–75.

Yancey, P.H. and Somero, G.N., 1978. Temperature dependence of intracellular pH: its role in the conservation of pyruvate apparent K_m values of vertebrates lactate dehydrogenases. *Journal of Comparative Physiology*, **125**: 129–134.

ACKNOWLEDGMENTS

CS and LDH assays were taught to Jose Torres by George Somero of Stanford University, who refined them and initially applied them to marine species as indicators of

metabolic status. Discussions with Pat Walsh and Liz Clarke of the University of Miami were instrumental in initially applying the techniques to individual plankters. The text has been distilled from our own experience as well as from numerous handouts and protocols that we have accumulated through the years. We gratefully acknowledge the help of our colleagues in passing on their knowledge, a tradition we are continuing here.

10.8 EXCRETION (T. Ikeda)

Live zooplankton release a variety of substances into the surrounding water. Those substances may be classified into solid forms (feces, molts for crustaceans, gelatinous 'houses' for appendicularians, etc.) and liquid forms. We will consider only the liquid forms in our treatment of 'excretion'. Zooplankton excretion has been measured as dissolved nitrogen (total-N, amino-N, urea-N and ammonia-N) and dissolved phosphorus compounds (total-P, organic-P and inorganic-P). Filtered sea water (see Washing/experimental sea water, section 10.7.1) has been used in most of the previous determinations of zooplankton excretion. From a technical viewpoint, the use of filtered sea water is simple in design and free from complicated corrections for simultaneous uptake/release of nitrogen or phosphorus by prey organisms. However, excretion rates determined on zooplankton placed in filtered sea water (i.e. no food) may provide conservative estimates (Feeding, section 10.3.1) Not only food, but various internal and external conditions of zooplankton are also known to affect the results (Technical problems, section 10.3.1). These methodological constraints need to be kept in mind for extrapolating laboratory-determined excretion data to field populations.

The two methods commonly used to measure excretion rates of marine zooplankton, are described in sections 10.8.1 and 10.8.2.

10.8.1 Single end-point method

Preparation of bottles and filtered sea water, and procedures of washing and incubation of zooplankters are fully described in Oxygen consumption – Winkler titration (T. Ikeda), section 10.7.1. Sample water withdrawn at the end of experiment is used for the analysis of nitrogen and phosphorus compounds instead of dissolved oxygen analysis. Excretion rate (E) of nitrogen and/or phosphorus compounds is calculated as;

$$\begin{aligned} E &= [(C_{t'} - C_0) - (C_t - C_0)] \times (V_c - V_z)/[t \times (N \text{ or } W)] \\ &= [(C_{t'} - C_t] \times (V_c - V_z)/[t \times (N \text{ or } W)] \end{aligned} \tag{10.23}$$

where C_0 is the concentration of the compounds at the beginning of incubation; C_t and $C_{t'}$ are the concentrations in control and experimental bottles respectively, at the end of incubation; V_c and V_z are the volumes of experimental bottles and zooplankton, respectively, t is the incubation time; N is the number of specimens, and W is the mass of specimens used. Note that C_0 is canceled out in the calculation. It is not necessary to determine it in the experiment in which filtered sea water is used. V_z is estimated from wet weight of specimens assuming 1 ml = 1 g wet weight, but is usually negligible.

E is thus expressed per individual or per unit weight (weight-specific rate) of zooplankters. Weight is often not mentioned in publications where the weight-specific

expression is used. In either expression of E, the weight of zooplankters should be provided with E so that other researchers can convert the results into both expressions.

Simultaneous analyses of dissolved oxygen and ammonia/inorganic phosphorus allow calculation of 'metabolic quotients' such as O:N (oxygen consumption:ammonia excretion), N:P (ammonia excretion:inorganic phosphate excretion) and O:P ratios (oxygen consumption:inorganic phosphate excretion) as indices of metabolic substrate (Metabolic quotient, section 10.3.3). This is an advantage of the 'Oxygen consumption – Winkler titration' method for excretion measurements.

10.8.2 Time-course method

The single end-point method described above assumes a constant excretion rate for the incubation period, but this may not always be the case. Time-course measurements can detect any changes in excretion rates during the course of an experiment. In a typical time-course measurement of ammonia and inorganic phosphate excretion, glass or polycarbonate bottles or beakers of appropriate volume may be used since the filling of sea water to the top of the container (i.e. no air space) is not necessary. These bottles or beakers should be cleaned with an acid solution (6NHCl). Containers are filled with a known volume (2.00 l in this example) of filtered sea water. Unlike the 'single end-point method' (Single end-point method, section 10.8.1), washing of specimens prior to incubation is not very critical in this method. After introduction of the zooplankton, each container is sealed with lids (for bottles) or parafilm (for beakers) to avoid contamination. The experiment is ready to run. A water bath with a temperature control unit is best for incubating the containers, which should also be kept in dark or dim light. During the course of the experiment, duplicate water samples for both ammonia and inorganic phosphate (10 ml $\times$ 4 = 40 ml) are withdrawn first at the start and then every 1 h up to 6 h. Because of this design, the volume of sea water in the container gradually declines during the course of the experiments. Since the density of specimens may affect the excretion rates (Container size/crowding, section 10.3.1), excessive depletion of sea water ($>70\%$ of the initial volume) is highly undesirable. A plot of ammonia or inorganic phosphate versus time will reveal whether the excretion rates are constant with time or not. Since the volume of sea water decreases during the course of an experiment using this method, the best way of expressing the data is not as concentration versus time but as actual amount per container (concentration $\times$ volume of sea water) versus time. When the increase of ammonia or inorganic phosphate with time is linear, excretion rate E can be calculated from the slope of the regression line (i.e. the increase in the amounts of ammonia or inorganic phosphate per unit of time) divided by the number of specimens or by the mass of specimens incubated.

10.8.3 Ammonia and inorganic phosphate analysis

The following is a slightly modified analytical method for ammonia and inorganic phosphate from the manuals of sea water analysis authored by Strickland and Parsons (1972) and Parsons *et al.* (1984). The sample water (50 ml) needed for the original method is reduced to 10 ml. Details of analytical methods for other nitrogen compounds (urea, dissolved free amino acids) and phosphorus compounds (total phosphorus, organic phosphorus) may also be found in these manuals.

AMMONIA-N

Reagents

1) Deionized water Remove the ammonia from distilled water by passing it through a small column of cation exchange resin in the hydrogen form just before use and store the water in a tightly stoppered glass flask.
2) Phenol solution Dissolve 20 g of crystalline phenol (analytical reagent grade) in 200 ml of 95% (v/v) ethanol.
3) Sodium nitroprusside solution Dissolve 1.0 g of sodium nitroprusside $Na_2Fe(CN)_5NO{\cdot}2H_2O$ in 200 ml of deionized water. The solution is stored in a brown bottle. It is stable for about a month.
4) Alkaline solution Dissolve 100 g of trisodium citrate and 5 g of sodium hydroxide (analytical reagent grade) in 500 ml of deionized water. This solution is stable for a long period.
5) Sodium hypochlorite solution Use a solution of commercial hypochlorite (e.g. Chlorox) which should be at least 1.5*N* (see Note a).
6) Oxidizing solution Mix 100 ml of reagent (4) and 25 ml of reagent (5). It is best to prepare this solution immediately before the analysis.

Analytical procedure

Add 10 ml of the sample water (see Note b) to a test tube with an accompanying screw cap and then 0.4 ml, 0.4 ml and 1 ml of solutions (2), (3) and (6) respectively. Mix well after each addition. Fit the cap to the test tube in order to avoid contamination from ammonia in the air and allow to stand at a temperature between 20 to 27 °C for 1 h. Then measure the extinction at 640 nm relative to distilled water in a spectrophotometer using 10-cm cells. It is best to conduct the reaction in a constant-temperature water bath. The reaction requires a full 60 min for completion. During that time the samples should never be placed in direct sunlight or near a window. The detrimental effect of sunlight on the reaction has been pointed out (Liddicoat *et al.* 1975).

Ammonia-N concentration of the sample water can be obtained from the following equation (see Note c):

$$\mu\text{g-at N l}^{-1} = (E - E_b) \times F \tag{10.24}$$

where E is the extinction of the sample water (a mean of duplicate readings), E_b is the extinction of reagent blank, and F is the factor. For E_b and F, see Calibration, section 10.8.3. 1 μg-at N is equivalent to 14 μg N.

Calibration

Dissolve 0.6607 g of ammonia sulfate (analytical reagent grade) in 1 l of deionized water (1 ml $\equiv$ 10 μg-at N). Add 1 ml of chloroform and store in a dark place with a stopper. At the time of use dilute the standard solution 100 times with distilled water to make a secondary solution, and dilute this solution further with filtered sea water containing as little ammonia as possible to make the standard solution. If sea water is added to 10 ml of this secondary solution to make 1 l of the standard solution, the resulting ammonia concentration is equivalent to 1.0 μg-at N l^{-1} of ammonia-N. Pipette 10 ml of dilute standard into each of three test tubes and carry out the ammonia determination described in Analytical procedure, section 10.8.2. Calculate the factor, F, as

$$F = 1.0/(E_{std} - E_b)$$

where E_{std} is the average extinction of three standards and E_b is the average extinction of

the reagent blank. To obtain E_b, use deionized water in place of dilute standard solution and carry out ammonia determination.

NOTES FOR THE AMMONIA ANALYSIS

a) To check on the strength of hypochlorite, dissolve 12.5 g of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 500 ml of distilled water. Add a few crystals (*ca.* 2 g) of potassium iodide (KI) to about 50 ml of distilled water in a small flask and pipette in 1.0 ml of hypochlorite solution. Add 5 to 10 drops of concentrated hydrochloric acid (HCl) and titrate the liberated iodine with the thiosulfate solution until no yellow color remains. Discard the hypochlorite when less than 12 ml of thiosulfate is used.

b) Temporary storage of sea water prior to analysis appears satisfactory in glass or polyethylene bottles, but analysis should not be delayed for more than 1 to 2 h at the most. If the analysis cannot be performed in this time period, samples should either be frozen at $-15\,^{\circ}C$ or stored unfrozen in the presence of 0.4 ml of phenol solution (Reagent (2)) per 10 ml of sample . Samples may be stored in either manner for up to 2 weeks (Degobbis 1973).

c) It assumes that the extinction is linear over the range of ammonia concentrations analyzed. For a linearity cheek of extinctions against ammonia concentration, a serial dilution of the standard solution should be made once during the experiment. The range of extinctions should cover the expected range of readings of experimental values.

INORGANIC PHOSPHATE-P

Reagents

1) Ammonium molybdate solution Dissolve 15 g of ammonium paramolybdate $(NH_4)\ Mo_7O_{24} \cdot 4H_2O$ (analytical reagent grade, preferably fine crystals) in 500 ml of distilled water. It should be protected from direct sunlight and be preserved in a polyethylene bottle. This solution is stable indefinitely.
2) Sulfuric acid solution Add 140 ml of concentrated sulfuric acid (analytical reagent grade, specific gravity of 1.8) to 900 ml of distilled water. Allow the solution to cool and preserve in a glass bottle.
3) Ascorbic acid solution Dissolve 27 g of good quality ascorbic acid in 500 ml of distilled water. Store the solution frozen in a polyethylene bottle. Thaw for use and refreeze at once. This solution can be kept for only about 1 week at room temperature but it is stable for several months if frozen.
4) Potassium antimonyl-tartrate solution Dissolve 0.34 g of good quality potassium antimonyl-tartrate $C_2H_2(OH)_2COOKCOO(SbO) \cdot 1/2H_2O$ in 250 ml of warm distilled water. The solution should be preserved either in a glass or polyethylene bottle. It is stable for several months.

Analytical procedure

Immediately before the analysis, the above solutions (1), (2), (3) and (4) are mixed in the ratio of 2:5:2:1 (v/v) respectively. Use this reagent for one-batch samples and discard any excess; it should not be kept for more than 6 h. To 10 ml of sample water (see Note a) in a test tube, add 1 ml of the mixed reagent and mix immediately. After 5 min or at most within 1 to 2 h, measure the extinction of the solution relative to distilled water in a 10-cm cell at a wavelength of 885 nm.

Phosphate-P concentration of the sample water can be obtained from the following equation (see Note b):

$$\mu\text{g-at P l}^{-1} = (E - E_b) \times F \tag{10.25}$$

where E is the average extinction of the sample water (a mean of duplicate readings), E_b is the average extinction of reagent blank, and F is the factor. For E_b and F, see Calibration, section 10.8.3. 1 μg – at P is equivalent to 31 μg P.

Calibration

Dissolve 0.816 g of anhydrous potassium dihydrogen phosphate KH_2PO_4 in 1 l of distilled water (1 ml $\equiv$ 6.0 μg-at P) and store in a dark bottle with 1 ml of chloroform. The solution is stable for many months. Dilute 10 ml of the standard solution to 1 l with distilled water (1 ml $\equiv 6.0 \times 10^{-2}$ μg-at P). Pipette 0.5 ml of dilute standard into each of three test tubes and make up to 10 ml with distilled water (3.0 μg-at P l^{-1}). Carry out the phosphate determination described in Analytical procedure, section 10.8.3. Calculate the factor, F, as

$$F = 3.00/(E_{std} - E_b) \tag{10.26}$$

where E_{std} is the average extinction of three standards and E_b is the average extinction of the reagent blank. To obtain E_b use distilled water in place of dilute standard and carry out the phosphate determination.

NOTES FOR THE INORGANIC PHOSPHATE ANALYSIS

a) As the storage of sample water for inorganic phosphate analysis is always associated with uncertain errors, immediate analyses are recommended (Gilmartin 1967).

b) It assumes the extinction is linear over the range of phosphate concentrations analyzed, and this is usually the case for phosphate analysis. For linearity check of extinctions against phosphate concentration, a serial dilution of the standard solution should be made once during the experiment. The range of extinctions should cover the expected range of readings of experimental values.

10.9 REFERENCES

Aarset, A.V. and Aunaas, T., 1990. Influence of environmental salinity on oxygen consumption and ammonia excretion of the arctic under-ice amphipod *Onisimus glacialis*. *Marine Biology*, **107**: 9–15.

Ahmed, S.I., Kenner, R.A. and King, F.D., 1976. Preservation of enzyme activity in marine plankton by low temperature freezing. *Marine Chemistry*, **4**: 133–139.

Alcaraz, M., Saiz, E. and Calbet, A., 1994. Small-scale turbulence and zooplankton metabolism: effects of turbulence on heartbeat rates of planktonic crustaceans. *Limnology and Oceanology*, **39**: 1465–1470.

Alldredge, A.L., Robinson, B.H., Fleminger, A., Torres, J.J., King, J.M. and Hamner, W.M., 1984. Direct sampling and *in situ* observation of a persistent copepod aggregation in the mesopelagic zone of the Santa Barbara Basin. *Marine Biology*, **80**: 75–81.

Anraku, M., 1964. Influence of the Cape Cod Canal on the hydrography and on the copepods in Buzzards Bay and Cape Cod Bay, Massachusetts. II. Respiration and feeding. *Limnology and Oceanography*, **9**: 195–206.

Aristegui, J. and Montero, M.F., 1995. The relationship between community respiration and ETS activity in the ocean. *Journal of Plankton Research*, **17**: 1563–1571.

Bailey, T.G., Torres, J.J., Youngbluth, M.J. and Owen, G.P., 1994. Effect of decompression on mesopelagic gelatinous zooplankton: a comparison of *in situ* and shipboard measurements of metabolism. *Marine Ecology Progress Series*, **113**: 13–27.

Båmstedt, U., 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. *Journal of Experimental Marine Biology and Ecology*, **42**: 267–283.

Båmstedt, U. and Tande, K.S., 1985. Respiration and excretion rates of *Calanus glacialis* in arctic waters. *Marine Biology*, **87**: 259–266.

Banse, K., 1982. Mass-scaled rates of respiration and intrinsic growth in very small invertebrates. *Marine Ecology Progress Series*, **9**: 281–297.

Barnes, H, 1959. *Apparatus and methods of oceanography*. Interscience, New York, 341 pp.

Beers, J.R., 1964. Ammonia and inorganic phosphorus excretion by the planktonic chaetognath, *Sagitta hispida* Conant. *Journal du Conseil Permanent International pour l'Exploration de la Mer*, **29**: 123–129.

Berges, J.A. and Ballantyne, J.S., 1991. Size scaling of whole-body maximal enzyme activities in aquatic crustaceans. *Canadian Journal of Fisheries and Aquatic Sciences*, **48**: 2385–2394.

Berges, J.A., Roff, J.C. and Ballantyne, J.S., 1990. Relationship between body size, growth rate, and maximal enzyme activities in the brine shrimp, *Artemia franciscanus*. *Biological Bulletin*, **179**: 287–296.

Berges, J.A. Roff, J.C. and Ballantyne, J.S., 1993. Enzymatic indices of respiration and ammonia excretion: relationships to body size and food levels. *Journal of Plankton Research*, **15**: 239–254.

Bidigare, R.R. and King, F.D., 1981. The measurement of glutamate dehydrogenase activity in *Praunus flexuosus* and its role in the regulation of ammonia excretion. *Comparative Biochemistry and Physiology*, **70B**: 409–413.

Biggs, D.C., 1977. Respiration and ammonium excretion by open ocean gelatinous zooplankton. *Limnology and Oceanography*, **22**: 108–117.

Black, E.G., Conner, A.R., Lam, K.-C. and Chiu, W.G., 1962. Changes in glycogen, pyruvate and lactate in rainbow trout (*Salmo gairdneri*) during and following muscular activity. *Journal of Fisheries Research Board of Canada*, **19**: 409–436.

Black, E.C., Manning, G.T. and Hayashi, K., 1966. Changes in levels of hemoglobin, oxygen, carbon dioxide, pyruvate, and lactate in venous blood of rainbow trout (*Salmo gairdneri*) during and following severe muscular activity. *Journal of Fisheries Research Board of Canada*, **23**: 783–795.

Brett, J.R., 1964. The respiratory metabolism and swimming performance of young sockeye salmon. *Journal of Fisheries Research Board of Canada*, **21**: 1184–1226.

Brightman, R.I., 1993. Energetics and RNA–DNA ratio in larval red drum *Sciaenops ocellatus*. PhD Thesis, University of South Florida, 173 pp.

Bulnheim, H.-P., 1972. Vergleichende Untersuchungen zur Atmungsphysiologie euryhaliner Gammariden unter besonderer Berucksichtigung der Salzgehaltsanpassung. *Helgoländer Wissenschaftliche Meeresuntersuchungen*, **23**: 485–534.

Buskey, E.J., 1998. Energetic costs of swarming behavior for the copepod *Dioithona oculata*. *Marine Biology*, **130**: 425–431.

Butler, E.I., Corner, E.D.S. and Marshall, S.M., 1969. On the nutrition and metabolism of zooplankton. VI. Feeding efficiency of *Calanus* in terms of nitrogen and phosphorus. *Journal of the Marine Biological Association of the United Kingdom*, **49**: 977–1001.

Butler, E.I., Corner, E.D.S. and Marshall, S.M., 1970. On the nutrition and metabolism of zooplankton. VIII. Seasonal survey of nitrogen and phosporus excretion by *Calanus* in the Clyde Sea area. *Journal of the Marine Biological Association UK*, **50**: 525–560.

Caperon, J., Schell, D., Hirota, J. and Laws, E., 1979. Ammonium excretion rates in Kaneohe Bay, Hawaii, measured by a ^{15}N isotope dilution technique. *Marine Biology*, **54**: 33–40.

Carpenter, J.H,., 1966. New measurements of oxygen solubility in pure and natural water. *Limnology and Oceanography*, **11**: 264–277.

Cetta, C.M., Madin, L.P. and Kremer, P., 1986. Respiration and excretion by oceanic salps. *Marine Biology*, **91**: 592–637.

Checkley, D.M., Dagg, M.J. and Uye, S., 1992. Feeding, excretion and egg production by individual and populations of the marine planktonic copepods *Acartia* spp. and *Centropages furcatus*. *Journal of Plankton Research*, **14**: 71–96.

Childress, J.J., 1971. Respiratory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens*. *Biological Bulletin*, **141**: 109–121.

Childress, J.J., 1975. The respiratory rates of midwater crustaceans as a function of depth of occurrence and relation to the oxygen minimum layer off southern California. *Comparative Biochemistry and Physiology*, **50A**: 787–799.

Childress, J.J., 1995. Are there physiological and biochemical adaptations of metabolism in deep-sea animals? *Trends in Ecology and Evolution*, **10**: 30–36.

Childress, J.J. and Mickel, T.J., 1985. Metabolic rates of animals from the hydrothermal vents and other deep-sea habitats. *Biological Society of Washington*, **6**: 249–260.

Childress, J.J. and Somero, G.N., 1979. Depth-related enzymic activities in muscle, brain, and heart of deep-living pelagic marine teleosts. *Marine Biology*, **52**: 273–283.

Childress, J.J. and Thuesen, E.V., 1993. Effect of hydrostatic pressure on metabolic rates of six species of deep-sea gelatinous zooplankton. *Limnology and Oceanography*, **38**: 665–670.

Childress, J.J., Barnes, A.T., Quetin, L.B. and Robinson, B.H., 1978. Thermally protected codends for the recovery of living deep-sea animals. *Deep-Sea Research*, **25**: 419–422.

Childress, J.J., Taylor, S.M., Cailliet, G.M. and Price, M.H., 1980. Patterns of growth, energy utilization and reproduction of some meso- and bathypelagic fishes off Southern California. *Marine Biology*, **61**: 27–40.

Christensen, J.P., Owens, T.G., Devol, A.H. and Packard, T.T., 1980. Respiration and physiological state in marine bacteria. *Marine Biology*, **55**: 267–276.

Clark, L.C., 1956. Monitor and control of blood and tissue oxygen tensions. *Transactions of American Society for Artificial Internal Organs*, **2**: 41–48.

Clarke, A., 1983. Life in cold water: the physiological ecology of polar marine ectotherms. *Oceanography and Marine Biology: an Annual Review*, **21**: 341–453.

Clarke, A., 1987. The adaptation of aquatic animals to low temperatures. In *The effects of low temperatures on biological systems*, pp. 315–348. B.W.W. Grout and G.J. Morris (eds), Edward Arnold, London, 500 pp.

Clarke, M.E. and Walsh, P.J., 1993. Effect of nutritional status on citrate synthase activity in *Acartia tonsa* and *Temora longicornis*. *Limnology and Oceanography*, **38**: 414–418.

Clarke, M.E., Calvi, C., Domeier, M., Edmonds, M. and Walsh, P.J., 1992. Effects of nutrition and temperature on metabolic enzyme activities in larval and juvenile red drum, *Sciaenops ocellatus*, and lane snapper, *Latjanus synagris*. *Marine Biology*, **112**: 31–36.

Conover, R., 1956. Oceanography of Long Island Sound, 1952–1954. VI. Biology of *Acartia clausi* and *A. tonsa*. *Bulletin of the Bingham Oceanographic Collection*, **15**: 156–233.

Conover, R.J., 1968. Zooplankton – life in a nutritionally dilute environment. *American Zoologist*, **8**: 107–118.

Conover, R.J., 1978. Transformation of organic matter. In *Marine Ecology Vol. 4, Dynamics*, pp. 221–499. O. Kinne (ed), John Wiley & Sons, New York, 746 pp.

Conover, R.J. and Corner, E.D.S., 1968. Respiration and nitrogen excretion by some marine zooplankton in relation to their life cycles. *Journal of the Marine Biological Association of the United Kingdom*, **48**: 49–75.

Conover, R.J. and Lalli, C.M., 1974. Feeding and growth in *Clione limacina* (Phipps), a pteropod mollusc. II. Assimilation, metabolism, and growth efficiency. *Journal of Experimental Marine Biology and Ecology*, **16**: 131–154.

Conover, R.J., Bedo, A.W. and Spry, A., 1988. Arctic zooplankton prefer living ice algae: a caution for zooplankton excretion measurements. *Journal of Plankton Research*, **10**: 267–282.

Corner, E.D.S. and Davies, A.C., 1971. Plankton as a factor in the nitrogen and phosphorus cycles in the sea. *Advances in Marine Biology*, **9**: 101–204.

Corner, E.D.S. and Newell, B.S., 1967. On the nutrition and metabolism of zooplankton. IV. The forms of nitrogen excreted by *Calanus*. *Journal of the Marine Biological Association of the United Kingdom*, **47**: 113–120.

Corner, E.D.S., Cowey, C.B. and Marshall, S.M., 1965. On the nutrition and metabolism of zooplankton. III. Nitrogen excretion by *Calanus*. *Journal of the Marine Biological Association of the United Kingdom*, **45**: 429–442.

Corner, E.D.S., Head, R.N. and Kilvington, C.C., 1972. On the nutrition and metabolism of zooplankton. VIII. The grazing of *Biddulphia* cells by *Calanus helgolandicus*. *Journal of the Marine Biological Association of the United Kingdom*, **52**: 847–861.

Corner, E.D.S., Head, R.N., Kilvington, C.C. and Pennycuick, L., 1976. On the nutrition and metabolism of zooplankton. X. Quantitative aspects of *Calanus helgolandicus* feeding as a carnivore. *Journal of the Marine Biological Association of the United Kingdom*, **56**: 345–358.

Culberson, C.H., 1991. Dissolved oxygen. In *WOCE Operations manual. Vol. 3: The observational program*, section 3.1: WOCE Hydrographic program, part 3.1.3. WHP Operations and methods. WHP Office Report WHPO 91–1/WOCE Report No. 68/91.

Curl, H.J. and Sandberg, J., 1961. The measurement of dehydrogenase activity in marine organisms. *Journal of Marine Research*, **1209**: 123–138.

Damkaer, D.M., Dey, D.B., Heron, G.A. and Prentice, E.F., 1980. Effects of UV-B radiation on near-surface zooplankton of Puget Sound. *Oecologia (Berlin)*, **44**: 149–158.

DeCoursey, P.J., 1983. Biological timing. In *The biology of Crustacea. Vol. 7, Behavior and ecology*, pp. 107–162. F.J. Vernberg and W.B. Vernberg (eds), Academic Press, New York, 338 pp.

Degobbis, D., 1973. On the storage of seawater samples for ammonia determination. *Limnology and Oceanography*, **18**: 146–150.

Dey, D.B., Damkaer, D.M. and Heron, G.A., 1988. UV-B dose/dose-rate responses of seasonally abundant copepods of Puget Sound. *Oecologia (Berlin)*, **76**: 321–329.

Donnelly, J. and Torres, J.J., 1988. Oxygen consumption of midwater fishes and crustaceans from the eastern Gulf of Mexico. *Marine Biology*, **97**: 483–494.

Drits, A.V., Pasternak, A.F. and Kosobokova, K.N., 1994. Physiological characteristics of the antarctic copepod *Cananoides acutus* during the late summer in the Weddell Sea. *Hydrobiologia*, **292/293**: 201–207.

Duval, W.S. and Geen, G.H., 1976. Diel feeding and respiration rhythms in zooplankton. *Limnology and Oceanography*, **21**: 823–829.

Fernández, F., 1977. The effect of the intensity of sunlight on the metabolic activity and feeding of several species of planktonic copepods. *Investigacion Pesquera*, **41**: 575–602.

Finlay, B.J., Span, A. and Ochsembein, G., 1983. Influence of physiological state on indices of respiration rate in Protozoa. *Comparative Biochemistry and Physiology*, **74A**: 211–219.

Forward, R. and Fyhn, H.J., 1983. Osmoregulation of the krill, *Meganyctiphanes norvegica*. *Comparative Biochemistry and Physiology*, **74A**: 301–305.

Frank, T.M. and Widder, E.A., 1994a. Evidence for behavioral sensitivity to near-UV light in the deep-sea crustacean *Systellaspis debilis*. *Marine Biology*, **118**: 279–284.

Frank, T.M. and Widder, E.A., 1994b. Comparative study of behavioral-sensitivity thresholds to near-UV and blue-green light in deep-sea crustaceans. *Marine Biology*, **121**: 229–235.

Gardner, W.S. and Paffenhöfer, G.A., 1982. Nitrogen regeneration by the subtropical marine copepod *Eucalanus pileatus*. *Journal of Plankton Research*, **4**: 725–734.

Gardner, W.S. and Scavia, D., 1981. Kinetic examination of nitrogen release by zooplankters. *Limnology and Oceanography*, **26**: 801–810.

Gaudy, R., 1973. Les variations saisonnieres de la respiration chez quatre especies de copepodes pelagiques de Golfe de Marseille. *Netherlands Journal of Sea Research*, **7**: 267–279.

Gaudy, R., 1974. Feeding four species of pelagic copepods under experimental conditions. *Marine Biology*, **25**: 125–141.

Gaudy, R. and Boucher, J., 1983. Relation between respiration, excretion (ammonia and inorganic phosphorus) and activity of amylase and trypsin in different species of pelagic copepods from an Indian Ocean equatorial area. *Marine Biology*, **75**: 447–460.

Geiger, S.P., Torres, J.J. and Kawall, H.G., 1996. The effect of the ice edge bloom on physiological condition of copepods in the Weddell Sea: results from biochemical assays. EOS 76: OS212.

Gerber, R.P. and Gerber, M.B., 1979. Ingestion of natural particulate organic matter and subsequent assimilation, respiration and growth by tropical lagoon zooplankton. *Marine Biology*, **52**: 33–43.

Gilmartin, M., 1967. Changes in inorganic phosphate concentration occurring during seawater sample storage. *Limnology and Oceanography*, **12**: 325–328.

Gnaiger, E., 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In *Polarographic oxygen sensors*, pp. 337–345. E. Gnaiger and H. Forstner, (eds), Springer-Verlag, Berlin. 370 pp.

Gnaiger, E. and Forstner, H., 1983. *Polarographic oxygen sensors*. Springer-Verlag, Berlin, 370 pp.

Goldman, J.C., Caron, D.A. and Dennett, M.R., 1987. Regulation of gross efficiency and ammonium regeneration in bacteria by substrate C:N ratio. *Limnology and Oceanography*, **32**: 1239–1252.

Gómez, M., Torres, S. and Hernández-León, S., 1996. Modification of the electron transport system (ETS) method for routine measurements of respiratory rates of zooplankton. *South African Journal of Marine Science*, **17**: 15–20.

Gorsky, G., Parazzoli, I. and Fenaux, R., 1987. Influence of temperature changes on oxygen uptake and ammonia and phosphate excretion, in relation to body size and weight, in *Oikopleura dioica* (Appendicularia). *Marine Biology*, **94**: 191–201.

Halcrow, K., 1963. Acclimation to temperature in the marine copepod, *Calanus finmarchicus* (Gunner). *Limnology and Oceanography*, **8**: 1–8.

Hargrave, B.T. and Geen, G.H., 1968. Phosphorus excretion by zooplankton. *Limnology and Oceanography*, **13**: 332–342.

Harris, E., 1959. The nitrogen cycle in Long Island Sound. *Bulletin of the Bingham Oceanographic Collection*, **17**: 31–65.

Hemmingsen, A.M., 1960. Energy metabolism as related to body size and respiratory surface, and its evolution. *Reports of the Steno Memorial Hospital and the Nordisk Insulinlaboratorium*, **9**: 1–110.

Hernández-León, S. and Gómez, M., 1996. Factors affecting the respiration/ETS ratio in marine zooplankton. *Journal of Plankton Research*, **18**: 239–255.

Hernández-León, S. and Torres, S., 1997. The relationship between ammonia excretion and GDH activity in marine zooplankton. *Journal of Plankton Research*, **19**: 587–601.

Hirche, H.J., 1983. Overwintering of *Calanus finmarchicus* and *Calanus helgolandicus*. *Marine Ecology Progress Series*, **11**: 281–290.

Hochachka, P.W and Somero, G.N., 1984. *Biochemical adaptations*. Princeton University Press, Princeton, New Jersey, 537 pp.

Holeton, G.F., 1974. Metabolic cold adaptation of polar fish: fact or artifact? *Physiological Zoology*, **47**: 137–152.

Ikeda, T., 1970. Relationship between respiration rate and body size in marine plankton animals as a function of the temperature of habitat. *Bulletin of Faculty of Fisheries, Hokkaido University*, **21**: 91–112.

Ikeda, T., 1974. Nutritional ecology of marine zooplankton. *Memoirs of Faculty of Fisheries, Hokkaido University*, **22**: 1–97.

Ikeda, T., 1977a. The effect of laboratory conditions on the extrapolation of experimental measurements to the ecology of marine zooplankton. II. Effect of oxygen saturation on the respiration rate. *Bulletin of Plankton Society of Japan*, **24**: 19–28.

Ikeda, T., 1977b. The effect of laboratory conditions on the extrapolation of experimental measurements to the ecology of marine zooplankton. IV. Changes in respiration and excretion rates of boreal zooplankton species maintained under fed and starved conditions. *Marine Biology*, **41**: 241–252.

Ikeda, T., 1985. Metabolic rates of epipelagic marine zooplankton as a function of body mass and temperature. *Marine Biology*, **85**: 1–11.

Ikeda, T., 1988. Metabolism and chemical composition of crustaceans from the Antarctic mesopelagic zone. *Deep-Sea Research*, **35**: 1991–2002.

Ikeda, T., 1989a. Are antarctic zooplankton metabolically more cold-adapted than arctic zooplankton? An intra-generic comparison of oxygen consumption rates. *Journal of Plankton Research*, **11**: 619–624.

Ikeda, T., 1989b. Estimated respiration rate of myctophid fish from the enzyme activity of the electron-transport system. *Journal of the Oceanographical Society of Japan*, **45**: 167–173.

Ikeda, T., 1992. Growth and metabolism of the hyperiid amphipod, *Themisto japonica* (Bovallius), reared in the fluctuating and constant temperatures in the laboratory. *Journal of Plankton Research*, **14**: 925–935.

Ikeda, T. and Dixon, P., 1982. Body shrinkage as a possible overwintering mechanism of the antarctic krill, *Euphausia superba* Dana. *Journal of Experimental Marine Biology and Ecology*, **62**: 143–151.

Ikeda, T. and Dixon, P., 1984. The influence of feeding on the metabolic activity of antarctic krill (*Euphausia superba* Dana). *Polar Biology*, **3**: 1–9.

Ikeda, T. and Hirakawa, K., 1998. Metabolism and body composition of zooplankton in the cold mesopelagic zone of the southern Japan Sea. *Plankton Biology and Ecology*, **45**: 31–44.

Ikeda, T. and Mitchell, A.W., 1982. Oxygen uptake, ammonia excretion and phosphate excretion by krill and other Antarctic zooplankton in relation to their body size and chemical composition. *Marine Biology*, **71**: 283–298.

Ikeda, T. and Skjoldal, H.R., 1980. The effect of laboratory conditions on the extrapolation

of experimental measurements to the ecology of marine zooplankton. VI. Changes in physiological activities and biochemical components of *Acetes sibogae australis* and *Acartia australis* after capture. *Marine Biology*, **58**: 285–293.

Ikeda, T. and Skjoldal, H.R., 1989. Metabolism and elemental composition of zooplankton from the Barents Sea during early Arctic summer. *Marine Biology*, **100**: 173–183.

Ikeda, T., Hing Fay, E., Hutchinson, S.A. and Boto, G.M., 1982. Ammonia and inorganic phosphate excretion by zooplankton from inshore waters of the Great Barrier Reef, Queensland. I. Relationship between excretion and body size. *Australian Journal of Marine and Freshwater Research*, **33**: 55–70.

Jawed, M., 1969. Body nitrogen and nitrogenous excretion in *Neomysis rayii* Murdoch and *Euphausia pacifica* Hansen. *Limnology and Oceanography*, **14**: 748–754.

Jawed, M., 1973. Effects of environmental factors and body size on rates of oxygen consumption in *Archaeomysis grebnitzkii* and *Neomysis awatschensis* (Crustacea: Mysidae). *Marine Biology*, **21**: 173–179.

Johnson, S.B. and Attramadal, Y.G., 1982. A simple protective cod-end for recovering live specimens. *Journal of Experimental Marine Biology and Ecology*, **61**: 169–174.

Karanas, J.J., Worrest, R.C. and Van Dyke, H., 1981. Impact of UV-B radiation on the fecundity of the copepod *Acartia clausii*. *Marine Biology*, **65**: 125–133.

Kaupp, S.E., 1987. The ontogenetic development of the metabolic enzymes citrate synthase and lactate dehydrogenase in the swimming muscles of larval and juvenile fishes: a scaling of locomotory power. MS Thesis, University of California, San Diego, 204 pp.

Kaupp, S.E. and Somero, G.N., 1989. Empirically determined metabolic scaling in larval and juvenile marine fish. *American Zoology*, **29**: 55a.

Kenner, R.A. and Ahmed, S.I., 1975. Measurements of electron transport activities in marine phytoplankton. *Marine Biology*, **33**: 119–127.

Kester, D.R., 1975. Dissolved gases other than CO_2. In *Chemical oceanography*, pp. 497–556. J.P. Riley and G. Skirrow (eds), Academic Press, London.

Kils, U., 1979. Performance of antarctic krill *Euphausia superba* at different levels of oxygen saturation. *Meeresforschung*, **27**: 35–48.

King, F.D. and Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. *Limnology and Oceanography*, **20**: 849–854.

Kinne, O., 1964. Non-genetic adaptation to temperature and salinity. *Helgoländer Wissenschaftliche Meeresuntersuchungen*, **9**: 433–458.

Kiørboe, T., Mohlenberg, F. and Hamburger, K., 1985. Bioenergetics of the planktonic copepod *Acartia tonsa*: relation between feeding, egg production and respiration, and composition of specific dynamic action. *Marine Ecology Progress Series*, **26**: 85–97.

Kremer, P., 1977. Respiration and excretion by the ctenophore *Mnemiopsis leidy*. *Marine Biology*, **44**: 43–50.

Kremer, P. and Reeve, M.R., 1989. Growth dynamics of a ctenophore (*Mnemiopsis*) in relation to variable food supply. II. Carbon budgets and growth model. *Journal of Plankton Research*, **11**: 553–574.

Kremer, P., Canino, M.F. and Gilmer, R.W 1986. Metabolism of epipelagic tropical ctenophores. *Marine Biology*, **90**: 403–412.

Lance, J., 1965. Respiration and osmotic behavior of the copepod *Acartia tonsa* in diluted sea water. *Comparative Biochemistry and Physiology*, **14**: 155–165.

Langdon, C., 1984. Dissolved oxygen monitoring system using a pulsed electrode: design, performance, and evaluation. *Deep-Sea Research*, **31**: 1357–1367.

Laws, E.A. and Archie, J.W., 1981. Appropriate use of regression analysis in marine biology. *Marine Biology*, **65**: 13–16.

Le Borgne, R.P., 1973. Etude de la respiration et de l'excretion mauritanien (mars–avril, 1972). *Marine Biology*, **19**: 249–257.

Le Borgne, R.P., 1979. Influence of duration of incubation on zooplankton respiration and excretion results. *Journal of Experimental Marine Biology and Ecology*, **37**: 127–137.

Le Borgne, R., 1982. Les facteurs de variation de la respiration et de l'excretion d'azote et de phosphore de zooplanckton de l'Atlantique intertropical oriental. II. Nature des populations zooplanctoniques et facteurs de milieu. *Oceanographie tropicale*, **17**: 27–44.

Le Borgne, R., 1986. The release of soluble end products of metabolism. In *The biological chemistry of marine copepods*, pp. 109–164. E.D.S. Corner and S.C.M. O'Hara (eds), Clarendon Press, Oxford, 349 pp.

Lehman, J.T., 1980. Release and cycling of nutrients between planktonic algae and herbivores. *Limnology and Oceanography*, **25**: 620–632.

Liddicoat, M.L., Tibitts, S. and Butler, E.I., 1975. The determination of ammonia in seawater. *Limnology and Oceanography*, **20**: 131–133.

Lowery, M.S., Roberts, S.J. and Somero, G.N., 1987. Effects of starvation on the activities and localization of glycolytic enzymes in the white muscles of the barred sand bass *Paralabrax acutifer*. *Physiological Zoology*, **60**: 538–549.

Lowry, P.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with a Folin phenol reagent. *Journal of Biological Chemistry*, **193**: 265–275.

Marshall, S.M., 1973. Respiration and feeding in copepods. *Advances in Marine Biology*, **11**: 57–120.

Marshall, S.M. and Orr, A.P., 1958. Some uses of antibiotics in physiological experiments in sea water. *Journal of Marine Research*, **17**: 341–346.

Marshall, S.M., Nicholls, A.G. and Orr, A.P., 1935. On the biology of *Calanus finmarchicus*. VI. Oxygen consumption in relation to environmental conditions. *Journal of the Marine Biological Association of the United Kingdom*, **20**: 1–28.

Martin, J.H., 1968. Phytoplankton–zooplankton relationships in Narragansett Bay. III. Seasonal changes in zooplankton excretion rates in relation to phytoplankton abundance. *Limnology and Oceanography*, **13**: 63–71.

Mayzaud, P., 1973. Respiration and nitrogen excretion of zooplankton. II. Studies of the metabolic characteristics of starved animals. *Marine Biology*, **21**: 19–28.

Mayzaud, P., 1976. Respiration and nitrogen excretion of zooplankton. IV. The influence of starvation on the metabolism and the biochemical composition of some species. *Marine Biology*, **37**: 47–58.

Mayzaud, P., 1986. Enzymatic measurements of metabolic processes concerned with respiration and ammonia excretion. In *The biological chemistry of copepods*, pp. 226–259. E.D.S. Corner and S.C.M. O'Hara (eds), Clarendon Press, Oxford, 349 pp.

Mayzaud, P. and Conover, R.A., 1988. O:N atomic ratio as a tool to describe zooplankton metabolism. *Marine Ecology Progress Series*, **45**: 289–302.

Mezykowski, T. and Rakusa-Suszczewski, S., 1979. The circadian rhythms in *Euphausia superba* Dana and its carbohydrate metabolism. *Meeresforschung*, **27**(1978/79): 124–129.

Miller, C.A. and Landry, M.R., 1984. Ingestion-independent rates of ammonium excretion by the copepod *Calanus pacificus*. *Marine Biology*, **78**: 265–270.

Morris, M.J., Kohlhage, K. and Gust, G., 1990. Mechanics and energetics of swimming in the small copepod *Acanthocyclops robustus* (Cyclopoda). *Marine Biology*, **107**: 83–91.

Mullin, M.M., Perry, M.J., Renger, E.H. and Evans, P.M., 1975. Nutrient regeneration by oceanic zooplankton: A comparison of methods. *Marine Science Communications*, **1**: 1–13.

Murray, C.N. and Riley, J.P., 1969. The solubility of gases in distilled water and sea water. II. Oxygen. *Deep-Sea Research*, **16**: 311–320.

Newell, R.C., Roy, A. and Armitage, K.B., 1976. An analysis of factors affecting the oxygen consumption of the isopod *Ligia oceanica*. *Physiological Zoology*, **49**: 109–137.

Newsholme, E.T. and Crabtree, B., 1986. Maximum catalytic activity of some key enzymes in provision of physiologically useful information about metabolic fluxes. *Journal of Experimental Zoology*, **239**: 159–167.

Niimi, A.J., 1978. Lag adjustment between estimated and actual physiological responses conducted in flow-through systems. *Journal of Fisheries Research Board of Canada*, **35**: 1265–1269.

Nival, P., Malara, G., Charra, R., Palazzoli, I. and Nival, S., 1974. Etude de la respiration et de l'excretion de quelques copepodes planktonique (Crustacea) dans la zone de remontee d'eau profonde des cotes Marocaines. *Journal of Experimental Marine Biology and Ecology*, **15**: 231–260.

Northby, J.A., 1976. A comment on rate measurements in open systems. *Limnology and Oceanography*, **21**: 180–182.

Omori, M., 1965. A 160 cm opening–closing plankton net. 1. Description of the gear. *Journal of the Oceanographical Society of Japan*, **21**: 212–220.

Omori, M., 1978. Some factors affecting dry weight, organic weight and concentration of carbon and nitrogen in freshly prepared and in preserved zooplankton. *Internationale Revue der Gesamten Hydrobiologie und Hydrographie*, **63**: 261–269.

Omori, M. and Ikeda, T., 1984. *Methods in marine zooplankton ecology*. John Wiley and Sons, New York, 323 pp.

Opalinski, K.W., 1979. Heartbeat rate in two antarctic crustaceans: *Euphausia superba* and *Parathemisto gaudichaudi*. *Polskie Archiwum Hydrobiologii*, **26**: 91–100.

Owens, T.G. and King, F.D., 1975. The measurement of respiratory electron-transport system activity in marine zooplankton. *Marine Biology*, **30**: 27–36.

Packard, T.T., 1969. The estimation of the oxygen utilization rate in seawater from the activity of the respiratory electron transport system in plankton. PhD Thesis, University of Washington, Seattle, 115 pp.

Packard, T.T., 1971. The measurement of respiratory electron transport activity in marine phytoplankton. *Journal of Marine Research*, **29**: 235–244.

Packard, T.T., 1985. Oxygen consumption in the ocean: measuring and mapping with enzyme analysis. In *Mapping strategies in chemical oceanography*, pp. 177–209. A. Zirino (ed.), American Chemical Society, Washington, DC, 467 pp.

Packard, T.T. and Taylor, P.B., 1968. The relationship between succinate dehydrogenase activity and oxygen consumption in the brine shrimp *Artemia salina*. *Limnology and Oceanography*, **13**: 552–555.

Packard, T.T. and Williams, P.J. LeB., 1981. Rates of respiratory oxygen consumption and electron transport in surface seawater from the northwest Atlantic. *Oceanological Acta*, **4**: 351–358.

Packard, T.T., Berdalet, E., Blasco, D. *et al.*, 1996. Oxygen consumption in the marine bacterium *Pseudomonas nautica* predicted from ETS activity and bisubstrate enzyme kinetics. *Journal of Plankton Research*, **18**: 1819–1835.

Packard, T.T., Devol, A.H. and King, F.D., 1975. The effect of temperature on the respiratory electron transport system in marine plankton. *Deep-Sea Research*, **22**: 237–249.

Paranjape, M.A., 1967. Molting and respiration of euphausiids. *Journal of Fisheries Research Board of Canada*, **24**: 1229–1240.

Park, Y.C., 1986a. Nitrogen regeneration and glutamate dehydrogenase activity of macrozooplankton in the southeastern Sea of Korea. *Journal of Oceanological Society of Korea*, **21**: 110–117.

Park, Y.C., 1986b. Impact of starvation and feeding experiments on ammonium excretion and glutamate dehydrogenase activity of zooplankton. *Korean Biochemical Journal*, **19**: 251–256.

Park, Y.C., Carpenter, E.J. and Falkowski, P.G., 1986. Ammonium excretion and glutamate dehydrogenase activity of zooplankton in Great South Bay, New York. *Journal of Plankton Research*, **8**: 489–503.

Parsons, T.R., Maita, Y. and Lalli, C.M., 1984. *A manual of chemical and biological methods of seawater analysis*. Pergamon Press, Oxford. 164 pp.

Pavlova, E.V., 1994. Diel changes in copepod respiration rates. *Hydrobiologia*, **292/293**: 333–339.

Pearcy, W.G. and Small, L.F., 1968. Effects of pressure on the respiration of vertically migrating crustaceans. *Journal of Fisheries Research Board of Canada*, **25**: 1311–1316.

Pearcy, W.G., Theilacker, G.H. and Lasker, R., 1969. Oxygen consumption of *Euphausia pacifica*. The lack of a diel rhythm or light–dark effect, with a comparison of experimental techniques. *Limnology and Oceanography*, **14**: 219–223.

Peterson, G.I., 1977. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Analytical Biochemistry*, **83**: 346–356.

Peterson, G.I., 1983. Determination of total protein. *Methods in Enzymology*, **91**: 95–119.

Pomeroy, L.R., Mathews, H.M. and Min, H.S., 1963. Excretion of phosphate and soluble organic phosphorus compounds by zooplankton. *Limnology and Oceanography*, **8**: 50–55.

Propp, M.V., Garber, M.R. and Ryabuscko, V.I., 1982. Unstable processes in the metabolic rate measurements in flow-through systems. *Marine Biology*, **67**: 47–49.

Prosser, C.L., 1961. Oxygen: respiration and metabolism. In *Comparative Animal Physiology*, pp. 165–211. C.L. Prosser and F.A. Brown, Jr (eds), W.B. Saunders, Philadelphia, 688 pp.

Quetin, L.B. and Mickel, T.J., 1983. Sealed respirometers for small invertebrates. In *Polarographic oxygen sensors*, pp. 184–189. E. Gnaiger and H. Forstner (eds), Springer-Verlag, Berlin, 370 pp.

Quetin, L.B., Ross, R.M. and Uchio, K., 1980. Metabolic characteristics of midwater zooplankton: ammonia excretion, O:N ratios, and the effect of starvation. *Marine Biology*, **59**: 201–209.

Rakusa-Suszczewski, S., McWhinnie, M.A. and Cahoon, M.O., 1976. Respiration of the Antarctic copepod, *Rhincalanus gigas*. *Limnology and Oceanography*, **21**: 763–765.

Raymont, J.E.G., 1983. *Plankton and productivity in the oceans. Vol. 2: Zooplankton*. Pergamon Press, Oxford, 824 pp.

Raymont, J.E.G. and Conover, R.J., 1961. Further investigations on the carbohydrate content of marine zooplankton. *Limnology and Oceanography*, **6**: 154–164.

Raymont, J.E.G. and Krisnaswamy, S., 1960. Carbohydrates in some marine planktonic animals. *Journal of the Marine Biological Association of the United Kingdom*, **39**: 239–248.

Raymont, J.E.G. and Krishnaswamy, S., 1968. A method for determining the oxygen uptake and carbon dioxide output in *Neomysis integer*. *Internationale Revue der Gesamten Hydrobiologie und Hydrographie*, **53**: 563–572.

Raymont, J.E.G., Krisnaswamy, S. and Tundisi, J., 1967. Biochemical studies on marine zooplankton: IV. Investigations on succinic dehydrogenase activity in zooplankton with special reference to *Neomysis integer*. *Journal du Conseil Permanent International pour l'Exploration de la Mer*, **31**: 164–169.

Razouls, S., 1972. Influence des conditions experimentales sur le taux respiratoire des

copepodes planktoniques. *Journal of Experimental Marine Biology and Ecology*, **9**: 145–153.

Redfield, A.G., Ketchum, B.H. and Richards F.A., 1963. The influence of organisms on the composition of seawater. In *The Sea*, Vol. 2, pp. 26–77. M.N. Hill (ed.), Interscience, New York, 554 pp.

Reeve, M.R., 1970. The biology of Chaetognatha. I. Quantitative aspects of growth and egg production in *Sagitta hispida*. In *Marine food chains*, pp. 168–189. J.H. Steele (ed.), Oliver and Boyd, Edinburgh, 552 pp.

Reeve. M.R., 1981. Large cod-end reservoirs as an aid to the live collection of delicate zooplankton. *Limnology and Oceanography*, **26**: 577–580.

Regnault, M., 1987. Nitrogen excretion in marine and fresh-water Crustacea. *Biological Reviews of Cambridge Philosophical Society*, **62**: 1–24.

Richards, F.A., 1965. Anoxic basins and fjords. In *Chemical oceanography*, Vol. 1, pp. 611–645. J.P. Riley and G. Skirrow (eds), Academic Press, London and New York.

Ricker, W.E., 1973. Linear regressions in fishery research. *Journal of Fisheries Research Board of Canada*, **30**: 409–434.

Rogers, C.G., 1927. *Textbook of comparative physiology*. McGraw-Hill, New York, 635 pp.

Saiz, E. and Alcaraz, M., 1992. Enhanced excretion rates induced by small-scale turbulence in *Acartia* (Copepoda: Calanoida). *Journal of Plankton Research*, **14**: 681–689.

Satomi, M. and Pomeroy, L.R., 1965. Respiration and phosphorus excretion in some marine populations. *Ecology*, **46**: 877–881.

Savenkoff, C., Packard, T.T., Rodier, M., Gerino, M., Lefevre, D. and Denis, M., 1995. Relative contribution of dehydrogenases to overall respiratory ETS activity in some marine organisms. *Journal of Plankton Research*, **17**: 1593–1604.

Schneider, G., 1990. A comparison of carbon based ammonia excretion rates between gelatinous and non-gelatinous zooplankton: implications and consequences. *Marine Biology*, **106**: 219–225.

Segal, E., 1970. Invertebrates. In *Marine ecology*, Vol. 1 (Part 1), pp. 159–211. O. Kinne (ed.), Wiley-Interscience, London, 681 pp.

Skjoldal, H.R., Båmstedt, U., Klinken, J. and Laing, A., 1984. Changes with time after capture in the metabolic activity of the carnivorous copepod *Euchaeta norvegica* Boeck. *Journal of Experimental Marine Biology and Ecology*, **83**: 195–210.

Smith, K.L. Jr, 1982. Zooplankton of a bathyal benthic boundary layer: *In situ* rates of oxygen consumption and ammonia excretion. *Limnology and Oceanography*, **27**: 461–471.

Smith, K.L. Jr and Teal, J., 1973. Temperature and pressure effects on respiration of thecosomatous pteropods. *Deep-Sea Research*, **20**: 853–858.

Somero, G.N. and Childress, J.J., 1980. A violation of the metabolism-size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-sized fish. *Physiological Zoology*, **53**: 322–337.

Strickland, J.D.H. and Parsons, T.R., 1972. A practical handbook of seawater analysis, 2nd edn. *Bulletins of the Fisheries Research Board of Canada*, **167**: 1–310.

Stuart, V., 1986. Feeding and metabolism of *Euphausia lucens* (Euphausiacea) in the southern Benguela current. *Marine Biology*, **30**: 117–125.

Sullivan, K.M. and Somero, G.N., 1983. Size- and diet-related variation in enzyme activity and tissue composition in sablefish, *Anoplopoma fimbria*. *Biological Bulletin*, **164**: 315–326.

Taguchi, S. and Ishii, H., 1972. Shipboard experiments on respiration, excretion, and grazing of *Calanus cristatus* and *C. plumchrus* (Copepoda) in the northern North Pacific. In *Biological oceanography of the northern North Pacific Ocean*, pp. 419–431. A.Y. Takenouchi, M. Anraku, K. Banse *et al.* (eds), Idemitsu Shoten, Tokyo, 626 pp.

Takahashi, M. and Ikeda, T., 1975. Excretion of ammonia and inorganic phosphorus by *Euphausia pacifica* and *Metridia pacifica* at different concentrations of phytoplankton. *Journal of Fisheries Research Board of Canada*, **32**: 2189–2195.

Teal, J.M., 1971. Pressure effects on the respiration of vertically migrating decapod Crustacea. *American Zoologist*, **12**: 571–576.

Teal, J.M. and Carey, F.G., 1967. Respiration of a euphausiid from the oxygen minimum layer. *Limnology and Oceanography*, **12**: 548–550.

Teal, J.M. and Halcrow, K., 1962. A technique for measurement of respiration of single copepod at sea. *Journal du Conseil Permanent International pour l'Exploration de la Mer*, **27**: 125–128.

Thuesen, E.V. and Childress, J.J., 1993a. Enzymatic activities and metabolic rates of pelagic chaetognaths: lack of depth-related declines. *Limnology and Oceanography*, **38**: 935–948.

Thuesen, E.V. and Childress, J.J., 1993b. Metabolic rates, enzymatic activities, and chemical composition of some deep-sea worms particularly *Nectonemertes mirabilis* (Nemertea: Hoplonemartinea) and *Poeobius meseres* (Annelida: Polychaeta). *Deep-Sea Research*, **40**: 935–951.

Thuesen, E.V. and Childress, J.J., 1994. Oxygen consumption rates and metabolic enzyme activities of oceanic California medusae in relation to body size and habitat depth. *Biological Bulletin*, **187**: 84–98.

Thuesen, E.V., Miller, C.B. and Childress, J.J., 1998. An ecophysiological interpretation of oxygen consumption rates and enzymatic activities of deep-sea copepods. *Marine Ecology Progress Series*, **168**: 95–107.

Torres, J.J. and Childress, J.J., 1983. Relationship of oxygen consumption to swimming speed in *Euphausia pacifica*. I. Effects of temperature and pressure. *Marine Biology*, **74**: 79–86.

Torres, J.J. and Somerõ, G.N., 1988. Vertical distribution and metabolism in Antarctic mesopelagic fishes. *Comparative Biochemistry and Physiology*, **90B**: 521–528.

Torres, J.J., Aarset, A.V., Donnelly, J., Hopkins, T.L., Lancraft, T.M. and Ainley, D.G., 1994. Metabolism of antarctic micronektonic Crustacea as a function of depth of occurrence and season. *Marine Ecology Progress Series*, **113**: 207–219.

Torres, J.J., Belman, B.W. and Childress, J.J., 1979. Oxygen consumption rates of midwater fishes as a function of depth of occurrence. *Deep-Sea Research*, **26**: 185–197.

Vanderploeg, H.A., Laird, G.A., Liebig, J.R. and Gardner, W.S., 1986. Ammonium release by zooplankton in suspension of heat-killed algae and an evaluation of the flow-cell method. *Journal of Plankton Research*, **8**: 341–352.

Vernon, H.M., 1896. The respiratory exchange of lower marine invertebrates. *Journal of Physiology*, **19**: 18–70.

Vidal, J., 1980. Physioecology of zooplankton. III. Effects of phytoplankton concentration, temperature, and body size on the metabolic rate of *Calanus pacificus*. *Marine Biology*, **56**: 195–202.

Webb, K.L. and Johannes, R.E., 1967. Studies of the release of dissolved free amino acids by marine zooplankton. *Limnology and Oceanography*, **12**: 376–382.

Webb, K.L. and Johannes, R.E., 1969. Do marine crustaceans release dissolved amino acid? *Comparative Biochemistry and Physiology*, **29**: 875–878.

Weiss, R.F., 1970. The solubility of nitrogen, oxygen, and argon in water and seawater. *Deep-Sea Research*, **17**: 721–735

Williams-Howze, J., 1997. Dormancy in the free-living copepod orders Cyclopoida, Calanoida, and Harpacticoida. *Oceanography and Marine Biology: an Annual Review*, **35**: 257–321.

Williams, R. and Poulet, S.A., 1986. Relationship between the zooplankton, phytoplankton, particulate matter and dissolved free amino acids in the Celtic Sea. I. Unstratified water conditions. *Marine Biology*, **90**: 279–284.

Winkler, L.W. 1888. Die Bestimmung des im Wasser gelösten Sauerstoffes und die Löslichkeit des Sauerstoffs im Wasser. *Berichte der Deutschen Chemischen Gesellschaft zu Berlin*, **21**: 2843–2855.

Wright, P.A., 1995. Nitrogenous excretion: three end products, many physiological roles. *Journal of Experimental Biology*, **198**: 273–281.

Youngbluth, M.J., Kremer, P., Bailey, T.G. and Jacoby, C.A., 1988. Chemical composition, metabolic rates and feeding behavior of the midwater ctenophore *Bathocyroe fosteri*. *Marine Biology*, **98**: 87–94.

Zeiss, F.R., 1963. Effects of population densities on zooplankton respiration rates. *Limnology and Oceanography*, **8**: 110–115.

Zeuthen, E., 1947. Body size and metabolic rate in the animal kingdom with special regard to the marine micro-fauna. *Compte Rendus des Travaux du Laboratorie Carlsberg, Serie Chimique*, **26**: 17–161.

Zeuthen, E., 1953. Oxygen uptake as related to body size in organisms. *The Quarterly Review of Biology*, **28**: 1–12.