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***Nephtys hombergii*, a free-living predator in marine sediments: energy production under environmental stress**

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Abstract *Nephtys hombergii* is a free-living, burrowing predator in marine sediments. The worm is, therefore, exposed to various environmental conditions which tube-dwelling polychaetes of the same habitat most likely do not encounter. The worms have to survive periods of severe hypoxia and sulphide exposure, while at the same time, they have to maintain agility in order to feed on other invertebrates. *N. hombergii* is adapted to these conditions by utilising several strategies. The species has a remarkably high content of phosphagen (phosphoglycocyamine), which is the primary energy source during periods of environmental stress. With increasing hypoxia, energy is also provided via anaerobic glycolysis ($pO_2 < 7$ kPa), with strombine as the main end-product. Energy production via the succinate pathway becomes important only under severe hypoxia (< 2 kPa), suggesting a biphasic response to low oxygen conditions which probably is related to the worm's mode of life. The presence of sulphide resulted in a higher anaerobic energy flux and a more pronounced energy production via glycolysis than in anoxia alone. Nevertheless, after sulphide exposure under anaerobic conditions of < 24 h, *N. hombergii* is able to recover completely. Although *N. hombergii* appears to be well adapted to a habitat with short-term fluctuations in oxygen and appearance of hydrogen sulphide, its high energy demand as a predator renders it likely to limit its survival in an environment with longer lasting anoxia and concomitant sulphide exposure.

Introduction

The polychaete *Nephtys hombergii* is a very common species occurring from the intertidal to continental shelf

depths, particularly in muddy or clayed sand (Rainer 1991). It has been recorded in the Mediterranean and eastern Atlantic north to the Barents Sea, including the North Sea and the western Baltic (Hartmann-Schröder 1996). It shows a relatively high tolerance to a wide range of salinities and is suggested as an important intermediate predator, feeding on small endobenthic invertebrates (Schubert and Reise 1986; Beukema 1987). As a sediment dweller without a permanent tube, *N. hombergii* is mostly found in reduced sediment areas such as the intertidal flats of the Wadden Sea, where it has to cope with restricted oxygen availability and concomitant sulphide exposure of up to 1 mM (Giere 1992; Thiermann et al. 1996).

Since even small sulphide concentrations inhibit the cytochrome *c* oxidase, the key enzyme of aerobic respiration (Bagarinao and Vetter 1990), sulphide is highly toxic for many aerobic species (Vismann 1991; Bagarinao 1992). For benthic polychaetes this could be a minor problem, since low oxygen concentrations only require a switch from aerobic to anaerobic metabolism, irrespective of the presence of hydrogen sulphide. Reliance on anaerobic metabolism, however, does not provide complete protection against the toxic effects of sulphide. Vismann (1990) showed that the survival of the polychaetes *Nereis diversicolor* and *Nereis virens* is reduced under anoxia in the presence of sulphide. In the lugworm *Arenicola marina* survivorship declined by nearly one-third after exposure to anoxia and sulphide (0.01 mmol l⁻¹ sulphide, Groenendaal 1980). Although *N. hombergii* regularly inhabits sulphidic sediments, nothing is known, to our knowledge, about the influence of sulphide on its energy production.

First investigations on the mode of anaerobic energy production in *Nephtys hombergii* were performed by Schöttler (1982a). It was demonstrated that *N. hombergii* generally follows the metabolic pathways exploited by many marine invertebrates (reviews Schöttler and Bennett 1991; Grieshaber et al. 1994). During the initial phase of anaerobiosis, succinate and, to a lesser degree, L-alanine are accumulated. Prolonged anaerobiosis re-

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sults in the formation of the short-chain fatty acids acetate and propionate which are mostly excreted into the water. Although *N. hombergii* has a high glycolytic capacity, no lactate dehydrogenase was detected. Instead, strombine and alanopine dehydrogenases were present in comparatively high activities (Schöttler 1982a). These opine dehydrogenases are known from many marine invertebrates (Grieshaber and Kreutzer 1986) for catalysing the reduction of pyruvate to either strombine (co-substrate glycine) or alanopine (co-substrate alanine). However, it remains unsolved which opine is accumulated as the end-product of glycolysis. Furthermore, information is lacking about the importance of phosphagen as an energy source.

In order to understand the strategies used by *Nephtys hombergii* to cope with different kinds of environmental stress, we performed experiments under various simulated in situ conditions. The main emphasis was to specify (1) the energy source used for short-term energy production, (2) the influence of hydrogen sulphide on the mode of anaerobic energy production and (3) the worm's ability to recover from anoxic sulphide treatment.

Materials and methods

Collection and maintenance

Specimens of *Nephtys hombergii* were collected from the tidal flats of Königshafen (Sylt, Germany) during periods of low tide. The worms were kept in well-aerated artificial sea water (29‰ S, 12 °C) in tanks containing a 2 cm layer of sand. Individuals were adapted to these conditions for a minimum of 5 d prior to their use in the experiments.

Experimental procedure

All experiments were performed at 12 °C. Immediately after the experiments, the worms were rapidly blotted dry, dropped into liquid nitrogen and stored at -70 °C until tissue extraction.

Hypoxia

To simulate environmental hypoxia, ten specimens of *Nephtys hombergii* were incubated in round flasks containing 450 ml artificial sea water (29‰ S) at various oxygen pressures (pO₂). The worms were kept under these conditions for a period of 24 h. Different pO₂ were obtained by equilibrating the water in the incubation flasks with a mixture of pure nitrogen and oxygen (AGA, Germany), delivered by a gas mixing pump (M 200a, Wösthoff, Bochum, Germany). In order to maintain a stable oxygen partial pressure, the flasks were bubbled constantly during the incubations.

Anoxia in the presence and absence of sulphide

In order to study the influence of sulphide on the anaerobic energy production, specimens of *Nephtys hombergii* (North Sea, 29‰ S) were exposed to anoxia and different sulphide concentrations (0.1 and 1 mmol l⁻¹). Round flasks were used in the experiments which were equipped with special fittings for gassing (Schöttler and Schroff 1976). The flasks were filled with 450 ml of artificial sea water (29‰ S, 12 °C) and a thin layer of fine sand. Anoxia was achieved by bubbling the flasks with pure nitrogen (AGA, Germany) for 60 min.

At the beginning of an experiment, ten individuals of *Nephtys hombergii* were placed into each flask. Gassing was continued for 5 min before the stop-cocks were closed. In a parallel set of experiments, sulphide stock solution was added to the flasks to reach a final concentration of 0.1 or 1 mmol l⁻¹ sulphide. Sulphide concentrations of the incubation media were measured at the beginning and the end of each experimental run (3, 6, or 24 h).

Recovery from sulphide treatment

After 24 h anoxic incubation in the absence or presence of hydrogen sulphide (1 mmol l⁻¹), the worms were transferred into small tanks (ten worms in each), which contained well-aerated sea water (29‰ S, 12 °C). After different periods of time (0, 0.5, 1, 3, 6, 24 and 48 h), specimens were removed from a tank and prepared for further analysis as described above.

Muscular activity

In order to achieve increased muscular activity as it would occur during extensive burrowing, worms were placed individually into small tanks with sea water (29‰ S), connected by electrodes. Electrical impulses (3.5 to 8.0 V, AC) evoked contraction of the whole worms. Stimulation was continued until the worms were exhausted and ceased responding.

Determination of anaerobic end-products

The frozen tissues were extracted as described by Schiedek and Schöttler (1990). Succinate concentration was estimated according to Michal et al. (1976). Strombine and alanopine levels were determined by HPLC (Völkel et al. 1995). Short-chain fatty acids were measured after steam distillation of the incubation water by gas chromatography (Kluytmans et al. 1975). The separation and determination of the most important free amino acids was performed via HPLC (Schiedek 1997). Phosphoglycocyanine was measured with a test similar to the phosphotaurocyanine assay described in Pörtner et al. (1979). The glycocyanine kinase, used in the test, was purified from the body wall of *Nereis virens* after Schöttler (1982b). The presence of further phosphagens was tested (phosphocreatine, phosphotaurocyanine and phospho-L-arginine) using the respective kinases. Measurement of glycogen was employed after Schöttler et al. (1990).

Preparation of sulphide stock solutions

Stock solutions of hydrogen sulphide were prepared by dissolving washed crystals of Na₂S × H₂O (7 to 9 molecules H₂O per mole NaS) in N₂-saturated sea water (29‰ S). Immediately before use in the experiments, the concentration of the stock was determined by an iodometric titration (Poethke 1973).

Determination of sulphide concentrations

The sulphide concentration in the incubation water was determined spectrophotometrically at 670 nm, using the methylene blue method (Gilboa-Garber 1971). The term "sulphide", as used in the context of the present study, is intended to include H₂S, HS⁻ and S²⁻.

Data analysis

All metabolite concentrations are given as mean values ± SD. Significance between anoxic and sulphide treatments was determined after Welch (Sachs 1988) at the *p* = 0.05 level.

Results

Hypoxia

Phosphoglycocyanine was the only phosphagen present in *Nephtys hombergii*. Under normoxic conditions it could be measured in concentrations of about $160 \mu\text{mol g}^{-1}$ dry wt. With decreasing oxygen concentrations it was degraded rapidly (Table 1). However, even under anoxic conditions, it was only depleted by about 60%. Anaerobic energy production via glycolysis, as evident from the production of strombine, started at a $p\text{O}_2$ of about 3.4 kPa and finally reached about $17 \mu\text{mol g}^{-1}$ dry wt. Alanopine was only found in concentrations $< 1 \mu\text{mol g}^{-1}$ dry wt. The accumulation of the amino acid alanine as well as the decrease of the aspartate pools also started at a $p\text{O}_2$ of about 3.4 kPa. Succinate, an indicator for the propionate pathway, first appeared at a $p\text{O}_2$ of 1.7 kPa. Its production was more pronounced ($20 \mu\text{mol g}^{-1}$ dry wt) at very low oxygen concentrations. Under these conditions, the short-chain fatty acids acetate and propionate also reached their highest levels ($17 \mu\text{mol g}^{-1}$ dry wt and $27 \mu\text{mol g}^{-1}$ dry wt, respectively).

Tolerance to anoxia and sulphide

After onset of anoxia, about $100 \mu\text{mol g}^{-1}$ dry wt phosphoglycocyanine were used within the first 3 h. Thereafter, no further reduction was recorded (Fig. 1). The presence of sulphide (up to 1 mmol l^{-1}) caused no significant difference in the utilisation of the phosphagen. Strombine, the end-product of anaerobic glycolysis, accumulated from the beginning of anaerobiosis. The extent of accumulation was dependent on the sulphide concentration. Higher sulphide concentrations resulted in a clearly higher strombine accumulation (Fig. 2). Furthermore, alanopine reached concentrations up to $8 \mu\text{mol g}^{-1}$ dry wt in the presence of sulphide. The production of succinate was somewhat enhanced during the first 6 h of anoxia in the presence of sulphide, while the accumulation and excretion of propionate was similar in both types of incubations (Fig. 2). The final succinate concentration (after 24 h) appeared to be lower in the presence of sulphide compared to anoxia

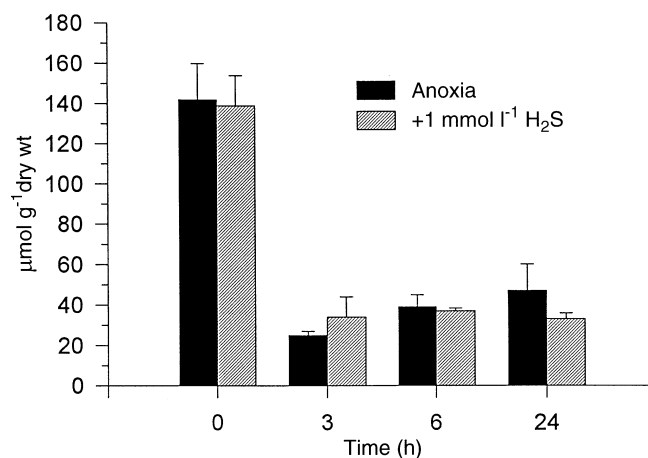


Fig. 1 *Nephtys hombergii*. Phosphoglycocyanine content ($\mu\text{mol g}^{-1}$ dry wt) after anoxic incubation (0 to 24 h) in absence and presence of sulphide (1 mmol l^{-1}). Mean \pm SD, $n = 3$

alone. A relationship between sulphide concentration and succinate/propionate production was not apparent.

Glycogen was stored at a concentration of about $120 \mu\text{mol g}^{-1}$ dry wt. Within 24 h of anoxia it was markedly degraded (Table 2). The presence of sulphide had no significant effect on the degradation of glycogen.

Recovery after exposure to anoxia and sulphide

The recovery from anoxic incubation in the absence or presence of sulphide (1 mmol l^{-1}) is reflected in the concentration changes of phosphoglycocyanine, succinate, alanopine and strombine. Most of the phosphoglycocyanine, depleted during anaerobiosis, was replenished within the first 3 h (Fig. 3a), while after sulphide exposure about 6 h were needed for its recovery. The accumulated succinate was metabolised immediately after the onset of aerobic conditions. About half of it disappeared within the first hour (anoxia) or 3 h (sulphide), respectively. The concentration of alanopine remained high for 6 h (anoxia). After exposure to sulphide, the alanopine concentration was significantly higher and oxidation started sooner after the onset of aerobic conditions (Fig. 3b). Within 1 h it had reached the same level as in the anoxic incubation without sulphide. The accumulation of strombine was nearly 50%

Table 1 *Nephtys hombergii*. Content of various metabolites ($\mu\text{mol g}^{-1}$ dry wt) after incubation (24 h) at different $p\text{O}_2$. Mean \pm SD, $n = 4$

$p\text{O}_2$ (kPa)	P-glycocyanine	Strombine	Aspartate	Alanine	Succinate	Propionate	Acetate
18	158 ± 16	< 0.5	35 ± 7	26 ± 4	< 0.5	0.7 ± 0.1	1.9 ± 0.2
13.8	142 ± 24	0.9 ± 0.1	32 ± 4	27 ± 4	< 0.5	0.6 ± 0.1	3.2 ± 0.2
6.9	167 ± 15	1.0 ± 0.1	31 ± 4	31 ± 7	< 0.5	0.5 ± 0.2	3.2 ± 0.3
3.4	131 ± 18	6.9 ± 2.7	30 ± 1	34 ± 5	< 0.5	1.6 ± 0.2	11.4 ± 6.0
1.7	109 ± 16	16.1 ± 5.0	21 ± 2	33 ± 9	8.7 ± 0.7	4.5 ± 1.3	10.4 ± 5.1
0.86	73 ± 9	15.6 ± 4.4	17 ± 3	43 ± 9	17.1 ± 2.7	6.2 ± 1.2	16.8 ± 6.6
0	67 ± 29	17.2 ± 2.5	14 ± 3	42 ± 5	20.3 ± 2.3	17.2 ± 2.9	27.4 ± 4.1

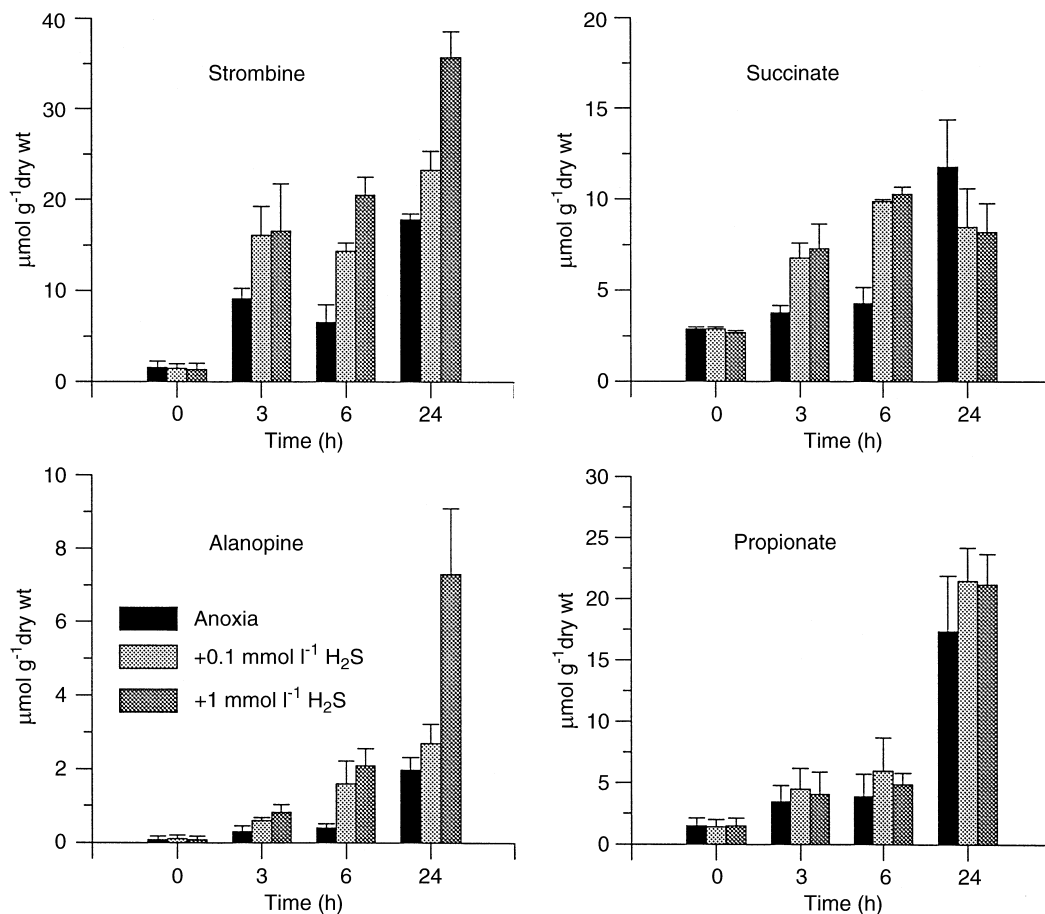


Fig. 2 *Nephtys hombergii*. Content of various metabolites ($\mu\text{mol g}^{-1}$ dry wt) after anoxic incubation (0 to 24 h) in absence and presence of sulphide (0.1 or 1 mmol l^{-1}). Mean \pm SD, $n = 3$

higher in the presence of sulphide. During recovery its concentration declined within the first 6 h to about $15 \mu\text{mol g}^{-1}$ dry wt. Similar concentrations as in the controls were finally reached after 48 h. In absence of sulphide, no decrease in the strombine concentration was detectable within the first 6 h of recovery. Nevertheless, after 24 h the concentration was nearly as low as in the controls.

Enhanced muscle activity

When the worms were stressed to exhaustion, about one-third ($40 \mu\text{mol g}^{-1}$ dry wt) of the phosphoglycocyanine was used (Table 3). Strombine and alanopine accumulated under these conditions to about $23 \mu\text{mol g}^{-1}$ dry wt and $4 \mu\text{mol g}^{-1}$ dry wt, respectively.

Table 2 *Nephtys hombergii*. Glycogen content ($\mu\text{mol glycosyl units g}^{-1}$ dry wt) after anoxic incubation in absence and presence of sulphide (1 mmol l^{-1}). Mean \pm SD, $n = 6$

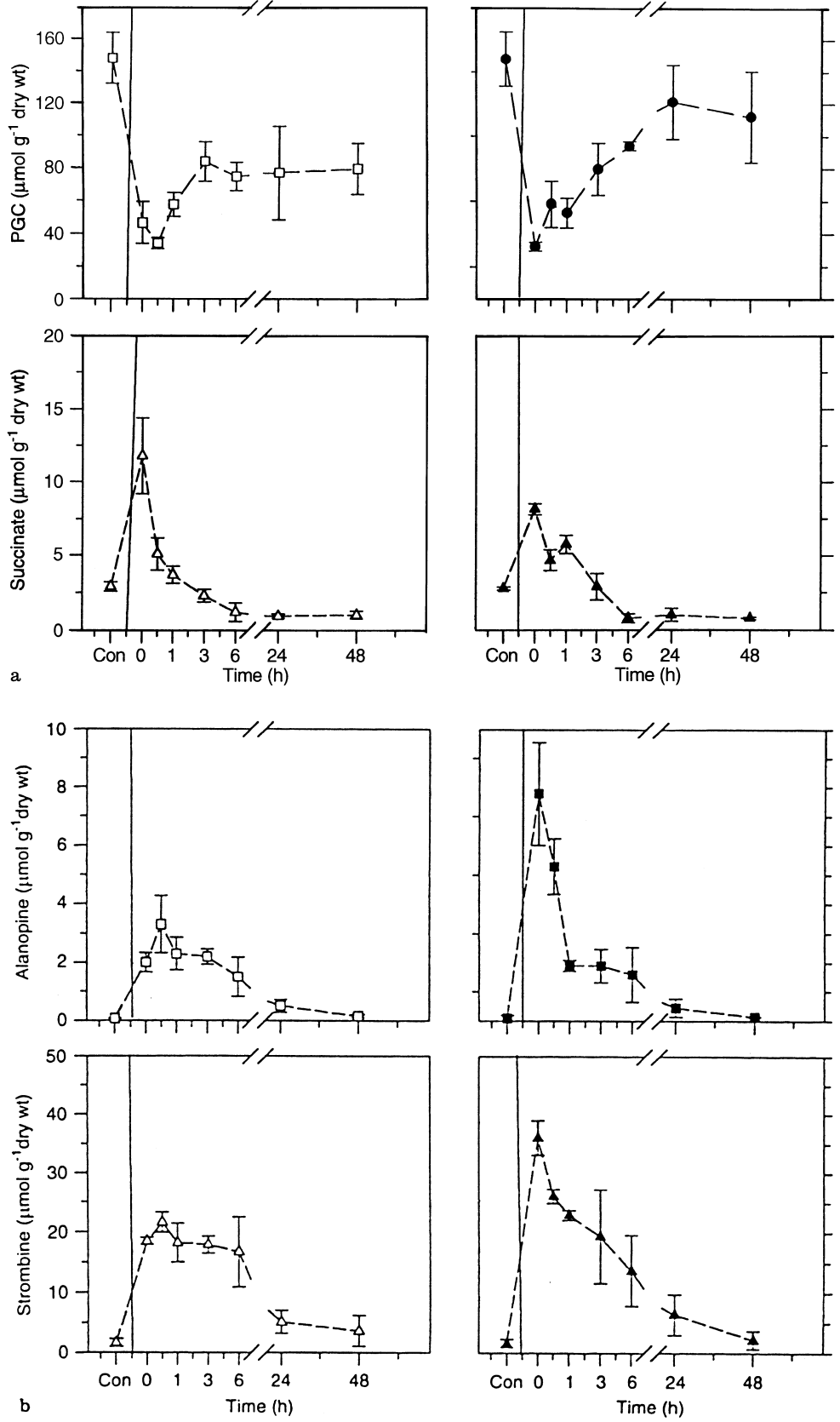
Incubation time (h)	Anoxia	Anoxia/H ₂ S
0	117 \pm 39	117 \pm 39
6	82 \pm 14	90 \pm 22
24	27 \pm 7	17 \pm 6

Discussion

Due to its predatory life habit, free-living in the sediments, the polychaete *Nephtys hombergii* is exposed to more adverse environmental conditions than tubedwelling species in the same environment. It has to provide energy to burrow and hunt, even under frequently anaerobic conditions, and even in the presence of the respiratory inhibitor sulphide.

The importance of phosphagens for immediate energy provision has been demonstrated for many marine invertebrates (Florkin and Scheer 1979), and differences in the type of phosphagen used have become obvious. Phosphoglycocyanine, as it was found in *Nephtys hombergii*, is also characteristic for *Scoloplos armiger* (Schöttler and Grieshaber 1988) and for polychaetes of the families Nereidae and Aphroditidae (Florkin and Scheer 1979). However, phosphagen concentrations as high as in *N. hombergii* have not been measured before in any other marine polychaete (Table 4). Comparably high concentrations have been detected only in the tail muscle of the shrimp *Crangon crangon*. This body part is specialised in performing maximal work during only one or

Fig. 3 *Nephtys hombergii*. Content of **a** phosphoglycocyanine (PGC) and succinate ($\mu\text{mol g}^{-1}$ dry wt) and **b** alanopine and strombine ($\mu\text{mol g}^{-1}$ dry wt) during aerobic recovery after anoxic incubation (24 h) in absence (*open symbols*) and presence (*closed symbols*) of sulphide (1 mmol l^{-1}). Mean \pm SD, $n = 3$ (*Con* aerobic controls)



two contractions, resulting in a nearly total depletion of the phosphagen (Onnen and Zebe 1983). *N. hombergii*, in contrast, reduced its phosphagen stores only to one-third during burst activity, although this worm also has to perform quick movements when burrowing through the sediment. Only the additional exposure to anoxia before performing enhanced muscular work, leads to a further depletion of the phosphoglycocyanine (Schiedek unpublished data). However, even these low values were still significantly higher compared to the phosphagen content of other marine polychaetes exposed to mere anoxia for 24 h (Table 4). The presence of sulphide appears to have no influence on the phosphagen utilisation in *N. hombergii*. Even after exposure to 1 mmol l⁻¹ sulphide, the phosphagen pool was not depleted as much as after anoxia and enhanced muscular work.

The quantitative importance of phosphagen as short-term energy provider in *Nephtys hombergii* becomes evident when calculating the share of phosphagen in the total anaerobic energy production (Table 4). This calculation reveals that in *N. hombergii* the phosphagen contributes to >20% of the total ATP production, mainly utilized in the beginning of anaerobiosis. Such a high share was only found in *Scoloplos armiger* which is like *N. hombergii* a free-living, burrowing polychaete (Table 4).

While phosphagen is a primary substrate for short-term anaerobic energy production, glycogen appears to be the major source during prolonged anaerobiosis. In polychaetes, the glycogen content can account for up to

35% of the dry weight. In the tube inhabiting *Nereis diversicolor*, for instance, it amounted to about 400 µmol g⁻¹ dry wt (Schöttler et al. 1990). In *Nephtys hombergii* as well as in *Scoloplos armiger* the glycogen pool appears to be relatively low at about 120 µmol g⁻¹ dry wt. Since both worms are known as very mobile animals, it is very likely that they use the faster break down of the phosphagen for gaining energy immediately, when dwelling through the hypoxic sediment.

Strombine is the main end-product of anaerobic glycolysis in *Nephtys hombergii* during environmental anaerobiosis as well as after enhanced muscular activity. Although the activities of the strombine dehydrogenase and the alanopine dehydrogenase were in the same range (Schöttler 1982a), alanopine accumulation was very low. A possible reason for the favoured accumulation of strombine could be the relatively high concentration of glycine in the worm's tissues (glycine/alanine ratio 10:1), the co-substrate in the reduction of pyruvate to strombine. Several studies on other marine invertebrates also indicate that it depends on the concentration of the corresponding amino acid which of the different opines is preferentially formed during environmental hypoxia (Kreutzer et al. 1989).

The production of strombine started in *Nephtys hombergii* already at a pO₂ < 3.4 kPa, when the worms were still able to use oxygen, as measurements of oxygen uptake have shown (Pederson 1991). The succinate pathway, in contrast, shows almost no importance for the energy production at this oxygen partial pressure. In other marine polychaetes, such as *Scoloplos armiger* (Schöttler and Grieshaber 1988) or *Arenicola marina* (Schöttler et al. 1983), succinate production and accumulation of glycolytic end-products simultaneously started to accumulate at the same oxygen partial pressure (critical pO₂). It is likely that, at this moderate hypoxia, aerobic energy production does not provide sufficient energy in *N. hombergii*. Therefore, the worm partially switches to anaerobic pathways, gaining additional ATP to meet its energy requirements through the

Table 3 *Nephtys hombergii*. Content of phosphoglycocyanine, strombine and alanopine (µmol g⁻¹ dry wt) after strong muscular activity. Mean ± SD

	P-glycocyanine	Strombine	Alanopine
Controls (n = 6)	147 ± 16	0.9 ± 0.3	<0.2
Burst activity (n = 10)	107 ± 30	22.6 ± 7.8	3.6 ± 0.5

Table 4 Phosphagen content (µmol g⁻¹ dry wt) in various polychaetes under normoxic conditions and after 24 h of anaerobiosis. For this calculation, the energy yield (production of ATP) of each end-product via the break-down of glycogen within 24 h of anoxia was estimated (alanine = 1.5; succinate = 2.5; acetate = 2.5;

propionate = 3.5; strombine = 1.5 µmol ATP µmol⁻¹ end-product). Utilisation of phosphoglycocyanine yields 1 µmol ATP (PGC P-glycocyanine; PC P-creatine; PTC P-taurocyanine; PA P-L-arginine)

Species, phosphagen	Normoxia	24 h anoxia	Share in anaerobic energy production (%)	Source
<i>Nephtys hombergii</i> , PGC	158 ± 16	67 ± 29	22	Present study
<i>Nereis diversicolor</i> , PGC/PC	66 ± 5	24 ± 3	9	Schöttler et al. (1990)
<i>Nereis virens</i> , PGC/PC	61 ± 6	18 ± 4	13	Schöttler unpubl.
<i>Scoloplos armiger</i> , PGC/PC	85 ± 7	15 ± 5	18	Schöttler and Grieshaber (1988)
<i>Arenicola marina</i> , PTC	45 ± 3	13 ± 2	11	Schöttler et al. (1990)
<i>Marenzelleria viridis</i> , PA	25 ± 3	7 ± 4	8	Schiedek (1997)

breakdown of phosphagen and accumulation of strombine. As a result, the energetic status of the cells ("energy charge", Atkinson 1977) should be balanced. Unfortunately, the determination of the adenosine nucleotides and, consequently, the calculation of the energy charge was not possible due to technical problems.

The presence of sulphide is likely to cause severe stress for sediment-dwelling polychaetes such as *Nephtys hombergii*. Especially the change of the pattern of anaerobic end-products shows that, under these conditions, *N. hombergii* is not able to reduce its energy requirements as much as under anoxia alone. The increased accumulation of strombine and alanopine after exposure to sulphide clearly indicates a higher glycolytic flux. Energy production via anaerobic glycolysis is less effective in terms of energy yield, when compared to the succinate pathway. This might explain the significantly reduced survivorship of *N. hombergii* after exposure to sulphidic anoxia, in comparison to specimens that experienced only anoxia (Arndt unpublished data). Very similar results were reported for *Nereis diversicolor* (Vismann 1990; Schiedek et al. 1996).

A fast recovery from hypoxia/anoxia is a mandatory requirement for a predator inhabiting sediments with periodically low oxygen content and concomitant appearance of hydrogen sulphide. Although *Nephtys hombergii* appears to be affected by hydrogen sulphide, this species is able to recover even after exposure to relatively high sulphide concentrations (1 mmol l⁻¹). However, oxidation of anaerobic metabolites was slower in the presence of sulphide than after anoxia alone. Nevertheless, in both cases succinate was metabolised and the phosphagen pool replenished within 3 to 6 h. This is only possible, if the cytochrome *c* oxidase is not inhibited by sulphide any longer. On the other hand, the depletion of the accumulated strombine and alanopine clearly took longer (about 24 h), suggesting that in *N. hombergii* the regeneration of the phosphagen is given metabolic preference. A very similar pattern of recovery from anoxia was found in *Scoloplos armiger* (Schöttler and Grieshaber 1988). This provides further evidence that actively burrowing polychaetes, relying on phosphagen as a primary energy source, have to refill these stores as fast as possible.

In summary, *Nephtys hombergii* shows a remarkably high content of phosphagen (phosphoglycocyamine), which is not completely exhausted even after short-term exposure to severe stress. It is suggested that this large phosphagen store is related to the worm's feeding strategy which requires energy very fast, even at times of severe oxygen deficiency. The results obtained from the anoxic incubations in the absence and presence of sulphide indicate that *N. hombergii* is well adapted to an environment with short-term fluctuations in oxygen levels and appearance of hydrogen sulphide such as the shallow tidal waters (Fallesen and Jørgensen 1991). Survival in an environment with longer lasting anoxia and sulphide exposure, as occur for instance in the deeper parts of the western Baltic (Weigelt 1991), is

likely to be poorer due to the worm's comparable high energy demand.

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