MUSSEL STRESS EXPERIMENT - FINAL REPORT

by

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This study was sponsored through the BMM-UGMM research contract nr. 85/17.

Both laboratories retain the responsability for the respective parts of the work they carried out. The general interpretation and discussion of the results was a cooperative effort.

Gent / Liège, 31-12-85

Summary.

A feasability study of an integrated pollution monitoring strategy using different tests of stress with the blue mussel, Mytilus edulis, was carried out in the Belgian coastal zone. The tests included physiological measurements, integrated in the scope for growth, and histological and chemical analyses of the digestive glands of the mussels: analysis of heavy metal content, biochemical characterization of metallothioneins, Shikata - test for histochemical demonstration of metalloproteins, lysosomal stability test and demonstration of NADPH - neotetrazolium reductase activity.

Native intertidal mussel populations were sampled in four stations along the Belgian coast. In addition mussels were transplanted to two experimental stations at sea (one at the west coast, one off the harbour of Zeebrugge). Each station had an upper cage (depth -3 m) and a lower cage (3m above the sediment).

In none of the stations we could show pollution stress due to heavy metals. Metallothioneins were not formed above the background level. In contrast, we found a varying degree of pollution due to organic compounds, as shown by the NTR measurements.

In the coastal stations pollution was most severe in the two harbours Zeebrugge and Oostende. This effect is apparent in scope for growth, NTR - reaction, and lysosomal stability. Relatively good conditions were found in Nieuwpoort. In Breskens scope for growth is relatively high, but the lysosomal stability is low, probably pointing to a combination of good food conditions and the presence of some stress factor.

In the experimental stations a clear pattern is found in NTR activity (which indicates organic pollution). The stress is higher in the upper cages than in the lower ones, implying a vertical gradient even in these shallow (10 m) stations. It is higher off Zeebrugge than off Nieuwpoort.

The station off Nieuwpoort has a higher scope for growth, and a higher realized shell growth than the station off Zeebrugge. Lysosomal stability is much influenced by a sometimes extensive tissue degeneration. We think this degeneration has developed in reaction to the heavy infestations with Mytilicola intestinalis, a parasitic - commensal copepod. Apparently this factor does not influence the scope for growth to the same extent as the lysosomal stability.

1. Introduction.

This report deals with the feasability of an integrated pollution monitoring strategy using different tests of stress with the blue mussel, Mytilus edulis. The strategy was mainly developed at the Institute for Marine Environmental Research (Plymouth, UK), and proposed by the "Water Research Centre" as a tool for the continuous monitoring of the Southern Bight of the North Sea and its estuaries. Specifically, we studied its utility as a pollution indicator in the Belgian coastal zone.

The method incorporates both physiological measurements at the whole organism level (integrated in the Scope For Growth) and histochemical analyses of the digestive gland.

1.1. Scope for growth.

Many environmental variables may influence the physiological condition of marine animals, among which are natural variables such as temperature (Widdows & Bayne, 1971). However, anthropogenic pollution stress (hydrocarbons, heavy metals, pesticides,...) may also exert a considerable influence on metabolism. They may be an important stress factor (Akberalli & Trueman, 1985), that can be measured by several physiological stress indicators: the Scope for Growth (Warren & Davis, 1967; Crisp, 1971; Widdows & Bayne, 1971; Thompson & Bayne, 1974), the growth rate (Crisp, 1971; Thompson & Bayne, 1974), the ratio of respired oxygen to excreted nitrogen (O:N ratio)(Corner & Cowey, 1968; Bayne, 1973; Widdows, 1978).

The Scope for Growth is defined as the energy that can be devoted to the production of somatic and reproductive tissues (Bayne et al., 1978). It is determined as the difference between the energy that is effectively assimilated and the energy lost through respiration and excretion. The rationale of integrating these energetic measurements in the Scope for Growth is that it provides an overall index of the metabolic activity, that can be correlated with the environmental conditions, including pollution stress. In fact, when an animal lives in a polluted environment it will have to use some energy for detoxification processes. Consequently, this energy will no longer be available for growth.

Several authors have studied the Scope for Growth of the mussel Mytilus edulis in relation to a multitude of environmental parameters: temperature (Widdows & Bayne, 1971; Bayne, 1973; Gabbott & Bayne, 1973), nutrition (Bayne, 1973; Gabbott & Bayne, 1973; Thompson & Bayne, 1974; Riisgard & Randlov, 1981; Navarro and Winter, 1982; Hawkins et al., 1985), suspended matter (Kiorboe et al., 1981), etc..

Widdows (1978a,b) has constructed a mathematical model relating Scope for Growth and its constituting factors to food quantity, mussel weight, and season.

1.2. Histochemical methods.

Apart from the scope for growth measurements we used three histochemical methods for the demonstration of pollution stress: the Shikata-test for metalloproteins, determination of the stability of lysosomes, and demonstration of NTR (NADPH-neotetrazolium reductase) activity. These methods were mainly developed by Moore and coworkers at IMER (Plymouth, UK) and are extensively described in Bayne et al. (1985).

The rationale behind the combination of these methods with the scope for growth measurements is double. Physiological measurements reveal integrated responses at the level of an entire organism. It is hypothesized that structural-functional alterations at the cellular level may manifest themselves earlier or at lower stress levels. Secondly, the different tests probe for different forms of pollution stress (e.g. heavy metals in the Shikata test, hydrocarbons and probably other organic compounds in the NTR test). It should therefore be possible to interpret an overall lowering of scope for growth in terms of specific pollution factors.

Bayne et al. (1985) thoroughly review the principles, methodology and results of the lysosomal destabilisation and NTR methods. We will only briefly summarize these aspects here. Extensive reference lists may also be found in Bayne et al. (1985).

The lysosomal-phagosomal complex is an intracellular system consisting of membranes to which (in normal conditions) mostly inactive hydrolytic enzymes are bound. Several stress factors may destabilize the lysosomal membranes, thus activating the hydrolytic enzymes which catabolize both cellular components and certain xenobiotics. Destabilization is preceded by sequestration and accumulation of the xenobiotics in the lysosomes. This process has been shown to operate with a large variety of compounds, e.g. viruses, aromatic hydrocarbons, metal ions etc.. Overloading of the storage capacity results in activation of the degradative enzymes.

The test for lysosomal stability is performed by destabilising the lysosomal membranes in an acid buffer solution (pH = 4.5) during succesively longer intervals (2, 5, 10, 15, 20, 25 min.). After destabilization a histochemical staining is performed for one of the lysosomal enzymes. Colour intensity is proportional to enzyme activity. As the enzymes themselves are also destabilized by the acid, a peak coloration is found at that time when the membranes have just been stabilized.

A decrease of the lysosomal destabilization time has been observed as a result of a wide variety of stress conditions: hyperthermia, hypoxia, starvation, spawning, injection of aromatic hydrocarbons and water-soluble crude oil fractions. The level of the response correlates well with the level of the stress stimulus. On the other hand, it correlates also with the physiological measurements (e.g. scope for growth).

NADPH-neotetrazolium reductase (NTR) is linked with the microsomal (smooth endoplasmatic reticulum) mixed-function oxidases. These enzyme systems play an important role in oxidizing (and detoxifying) toxic organic compounds in the cytoplasm. NTR represents the NADPH-oxidizing activity of the microsomal respiratory chains terminating in cytochrome P-450. Experiments have shown that its activity is stimulated by aromatic hydrocarbons, phenorbital, and the water-soluble fraction of crude oil. It is believed that a stimulation of its activity results only from this group of stressors.

The Shikata - test for metalloproteins (Shikata et al., 1974) is somewhat less validated than the other two methods. An acid permanganate solution is used (on fixed sections) to oxidise sulphydryl groups in metal-binding proteins, thereby removing the metal ions. The sulphonic acid residues remaining are stained by orcein, forming blue - purple granules (Jain et al. 1978). Staining is recorded on presence - absence basis of these granules. It should be noted that in contrast to the claims in some papers (e.g. Johnson et al., 1984) this method is not specific for metallothioneins. In principle all metal-binding proteins (and in addition other compounds, such as elastic fibres) are stained. One can only expect a quantitative difference between metallothioneins and other proteins.

Relatively high concentrations of heavy metals, well above the recorded total concentrations in the Belgian coastal zone, are needed to activate the formation of metallothioneins above the background level in M. edulis.

1.3. Sampling and the experimental setup.

The observations and experiments described here are of two types. First, we sampled simultaneously four native intertidal populations of M. edulis along the Belgian coast. Secondly, we set out three offshore buoys in the coastal zone. Two cages (at different depths) containing 50 mussels originating from the Eastern Scheldt (S.W. Holland) commercial cultures were attached to each buoy. After 52 days the mussels were recovered at two stations (unfortunately, the third one was lost). For a report of the cruises, see annex 1. The condition of these mussels was compared to the condition of the mussels prior to the experiment (except for scope for growth measurements, see below) and internally between the two recovered stations.

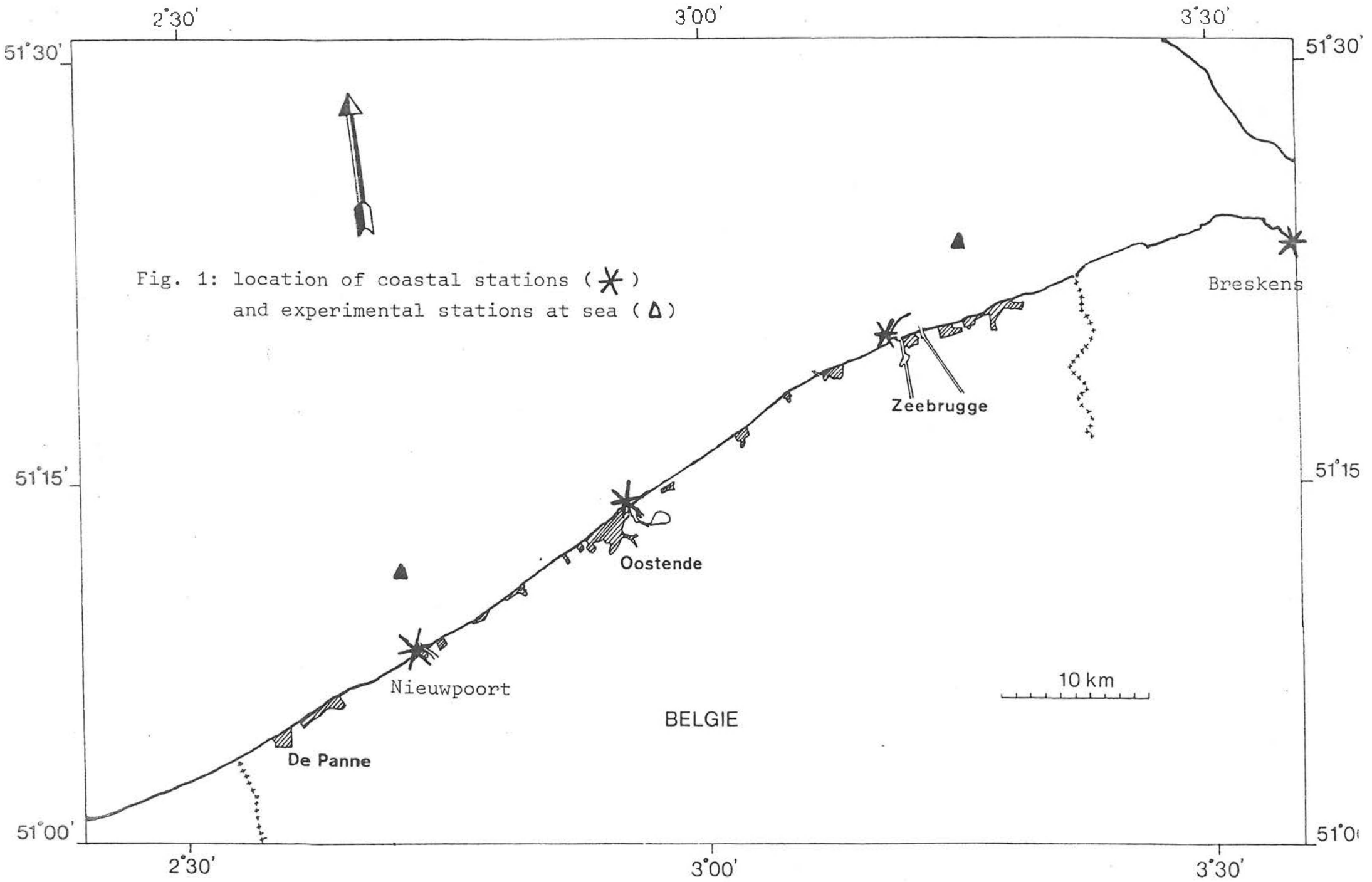
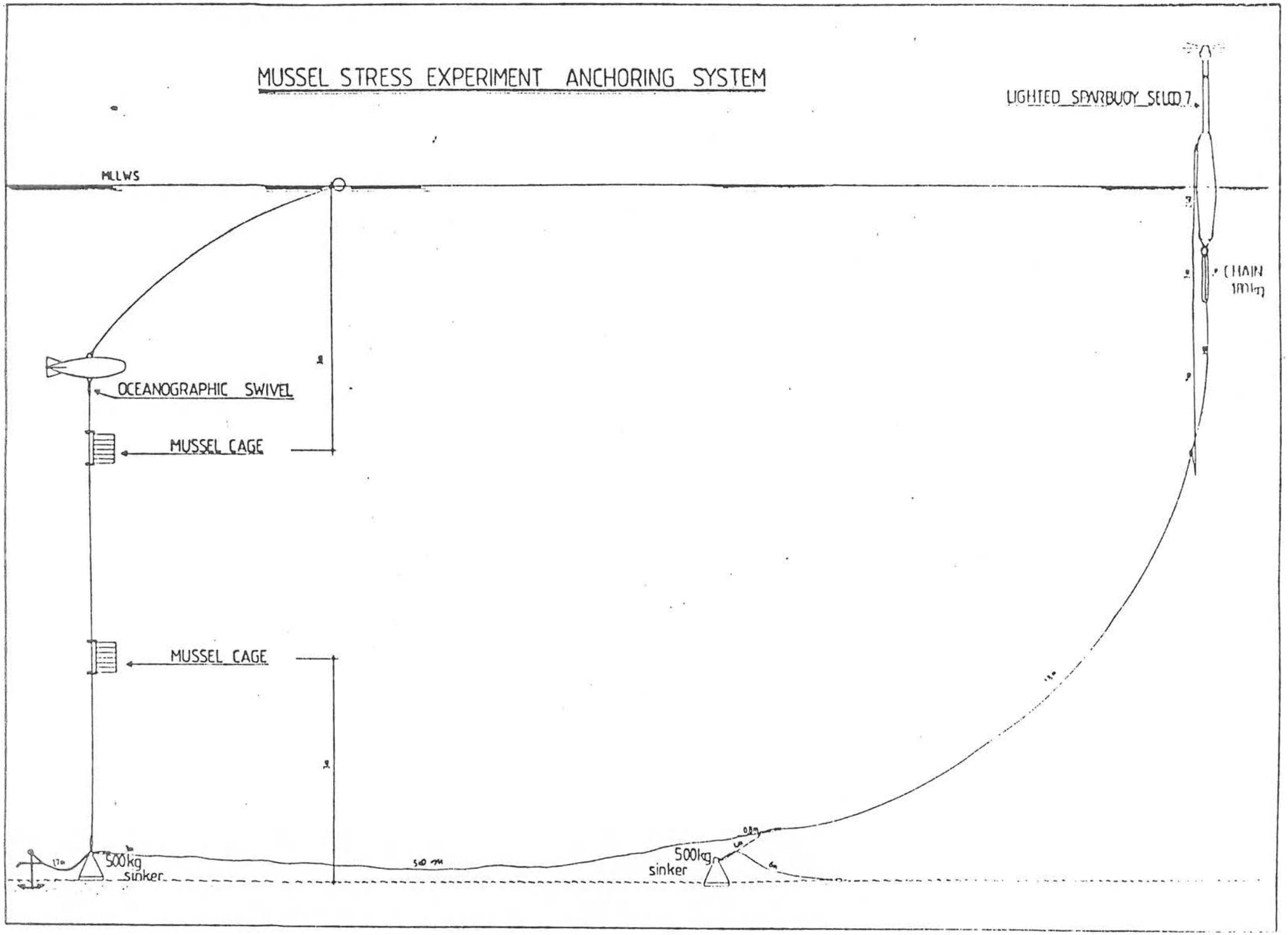


Fig. 1: location of coastal stations (✳)
and experimental stations at sea (Δ)

MUSSEL STRESS EXPERIMENT ANCHORING SYSTEM



2. MATERIAL AND METHODS.

2.1. Sampling sites and experimental setup.

We sampled native intertidal mussel populations on September 17, 1985 at four stations (Breskens, Zeebrugge, Oostende and Nieuwpoort : see map in Fig. 1) within half an hour before or after low tide .The largest possible animals were collected in the deepest accessible places. The precise locations are:

- Breskens: clumps of mussels were collected from a very old wreck protruding about 0.5 m from the sand, just east of the yacht harbour.
- Zeebrugge: mussels were collected from crevices between the rocks serving as surf-breakers at the west side (= sea-ward side) of the harbour pier.
- Oostende: mussels were collected on the surf-breaker nearest to the harbour. This surf-breaker protects a small beach, known to be heavily polluted.
- Nieuwpoort: mussels were collected from a surf-breaker located approximately 800 m west of the harbour.

At each site 10 mussels were collected and prepared for histochemical analysis; 10 mussels were also used for the scope for growth measurements. The remainder were used for the determination of heavy metal content and the biochemical analysis of metalloproteins.

The map in Fig. 1 also shows the location of the three experimental stations. Station 2 was lost, due to the breakage of a cable upon recovery. Fig. 2 schematically shows the mouring system. Two cages were attached to the cable connecting the subsurface buoy to the concrete block: one at depth -3 m, one at 3 m above the bottom. As the water depth was limited (Station off Zeebrugge: 9.3 m; Station off Nieuwpoort: 10.4 m), these cages were about 3-4 m apart. The 4 cages will be denoted by the following abbreviations:

N up : upper cage, station off Nieuwpoort
N lo : lower cage, station off Nieuwpoort
Z up : upper cage, station off Zeebrugge
Z lo : lower cage, station off Zeebrugge

The cages were constructed from plastic-coated metal wire (1 cm2 maze width). They were subdivided in 10 compartments, each containing 5 mussels.

The mussels used for this experiment were purchased from a commercial mussel culture at Yerseke (The Netherlands). They were freshly fished, not watered, and not treated in the machines that clear fouling organisms from the shells. Due to a defect in the circulating sea water system of the research vessel, the mussels were kept in moist air and at +- 10 °C during 15 (Station 1) to 20 h (Station 3). This bad treatment may have caused considerable mortality, and should be avoided in the future.

Ten mussels were fixed for histochemical analysis within 2 h after purchase. However, for practical reasons, the scope for growth measurements could only start the next day, implying the same harsh treatment as for the experimental mussels.

2.2. Scope For Growth measurements.

The experimental protocol for the scope for growth measurements has been described by numerous authors (Widdows & Bayne, 1971; Thompson & Bayne, 1974; Bayne et al., 1978; Widdows, 1978b; Widdows et al., 1981; Navarro & Winter, 1982; Hawkins et al. 1985). We have closely followed the method described by WRC Environment (1985).

Respiration rate is determined on individual mussels placed in closed containers, filled with 500 ml filtered sea water. The sea water is saturated with oxygen prior to the experiment. Oxygen consumption is measured with an oxygen electrode (Dissolved Oxygen Probe Model no. 8012-100) during approximately one hour. It is expressed in J/h by the conversion equation:

$$R = O_2 \text{ consumed (mg/h)} \times 14.23 \text{ J/mg}$$

(Crisp, 1971).

Ammonia excretion is directly measured by placing individual animals in 250 ml flasks containing filtered sea water. A control flask with sea water, but without an animal is run along. After approximately 3 h ammonia concentration is determined with the phenol-hypochlorite method of Solarzano, 1969). The energetic equivalent of the ammonia excretion is calculated as (Elliot & Davison, 1975) :

$$E = (NH_4\text{-}N) \text{ excreted (mg/h)} \times 24.8 \text{ (J/mg)}$$

Assimilation rate is measured form the "clearance rate" of suspension of planktonic algae : Phaeodactylum tricornutum Bohlin (Bacillariophyceae). A monospecific culture was established in the laboratory, based on a spike obtained from the Universite Catholique de Louvain la Neuve. The culture medium was that described by Guillard & Ryther (1982).

For the measurement of clearance rate the animals are placed in a flow-through system through which filtered sea water (with the algal suspension added), flows at a known rate (> 150 ml/min). The density of the algal cells is measured before and after their passage through the experimental chamber with a Turner Fluorometer Model 111. The fluorimetric measurements were previously calibrated with direct counts of the algal cells (see interim report of the ULg). The following formula then yields the clearance rate:

$$CR = \frac{CI - Co}{CI} \times Fl \qquad (l/h)$$

where CI = algal concentration before passage through the experimental chamber (no. of cells/ml).
Co = algal concentration after passage through the experimental chamber (no. of cells/ml).
Fl = flux (l/h).

In energetic units we have for the energy consumption rate:

$$C = CR \times (N) \times (16.3) \qquad (J/h)$$

where (N) = quantity of organic matter ingested (mg).
16.3 = energetic content of the algae (J/mg) as determined by Bayne et al. (1978) and Widdows (1978b).

The assimilation rate is subsequently calculated as the product of the consumption rate and the absorption efficiency. The latter is defined by the Conover (1966) ratio:

$$\text{Absorption efficiency} \mp e \mp \frac{F - E}{F\,(1 - E)}$$

where F = percentage of organic matter in the food
E = percentage of organic matter in the faeces

The assimilation is thus calculated as $A = C \times e$.

The scope for growth is then defined as:

$$P = A - (R + E) \qquad (J/h)$$

A minimum of 10 mussels was used for the scope for growth measurements in each station.

For the details of the experimental methods we refer to the interim report "Mussel stress experiment - Scope for Growth", Contrat UGMM-ULg Ref 85/16.

2.3. Histochemical methods.

10 um thick transverse (cryostat) sections of the digestive gland were cut with a cryostat American Opticals (hand-operated, cabinet temperature < - 25 C). The tissues were fixed in aromate-free frozen hexane, cooled in liquid N2. Size limitations of the cryostat tissue holders allowed for maximum three mussels to be cut together, and placed on the same object glass.

The mussels from the coastal populations were dissected upon transfer to the laboratory in damp air at ambient temperature (duration: 1-3 h between collection and fixation). The mussels from the offshore experiments were fixed aboard the ship, immediately after recovery of the cages.

For the histochemical stainings we followed very closely the protocols, established by IMER and WRC, which are given in App. I. We will mention here only a few relevant details.

+ Fixation of the sections for the Shikata-test was not done by submersion in Baker's formol. Instead the sections were placed in a closed container at 55 C for 2 h, together with a sufficient amount of formaldehyde in powder form. This method gives better fixation (Moore, pers. comm.) and prevents loosening of the sections from the object glasses.

+ Orcein (of which several forms exist) was purchased from UCB.

+ The destabilisation of the lysosomal membranes can be detected by staining for either of three different lysosomal enzymes: N-acetyl-β-hexosaminidase, β-glucuronidase and arylsulfatase. In principle the three methods should yield the same results, since all three enzymes are activated upon destabilization of the membranes. We have tried the three methods for two series of mussels, and could confirm that the results are identical. The staining of N-acetyl-β-hexosaminidase gives by far the most intense staining. This enzyme is the most prevalent of the three in M. edulis (Moore, pers. comm.). Therefore this method was adopted for the rest of the samples.

+ The stability of the colour is good for the Shikata-test and the lysosomal stability test. However, the purple-blue colour of the NTR - test is only stable when the section is mounted in a "UV-free water-soluble mounting medium" (DIFCO). The colour reacted very quickly with all other mounting media we tried. Unfortunately, we had to wait 3 months between ordering and delivery of the good mounting medium. For that reason we have only relative comparisons of the NTR-reaction between the coastal populations (from immediate inspections) but we are not able to compare them with the experimental stations.

+ The scaling of the NTR-reaction on a 0-5 scale proved to be relatively difficult. We have been able to order the different stations in decreasing order of intensity of staining (two observers arriving independently at the same ordering) but are not able to define the scale so that unequivocal scaling of the individual sections is possible in this way. A test has been performed with an automatic image-analyser by P. Aerts and W. Decoster (image-analysis centre, RUG). It is possible with this method to separate the blue - purple colour from the background, and to measure the intensity and extent of staining objectively. A measurement will be performed on all sections as soon as the time schedule of the image analysis centre will permit it. (planned early February - the results will be communicated in a separate short note).

+ A general staining of the sections with the Papanicoloau method (see Bayne et al., 1985 : p. 183 for a protocol) was performed. These sections were used to measure the proliferation of an aberrant connective-type tissue, which we suppose developed in reaction to infestation by Mytilicola intestinalis, a parasitic - commensal copepod. The proliferation of this tissue was expressed as the % of the surface of normal tissue (with digestive tubuli) and degenerative tissue that was occupied by the latter. Surfaces were meassured with a planimeter on drawings made with a camera lucida.

3. RESULTS.

3.1. Measurements on the whole organism level.

3.1.1. Mortality of mussels exposed in experimental stations at sea

After the 52 days of exposure at sea, the mortality of the mussels has been recorded in each cage. The results are shown in Fig. 3. The highest mortality was observed in the cage Z lo, the lowest in the N up cage:

Zlo > Nlo > Zup > Nup

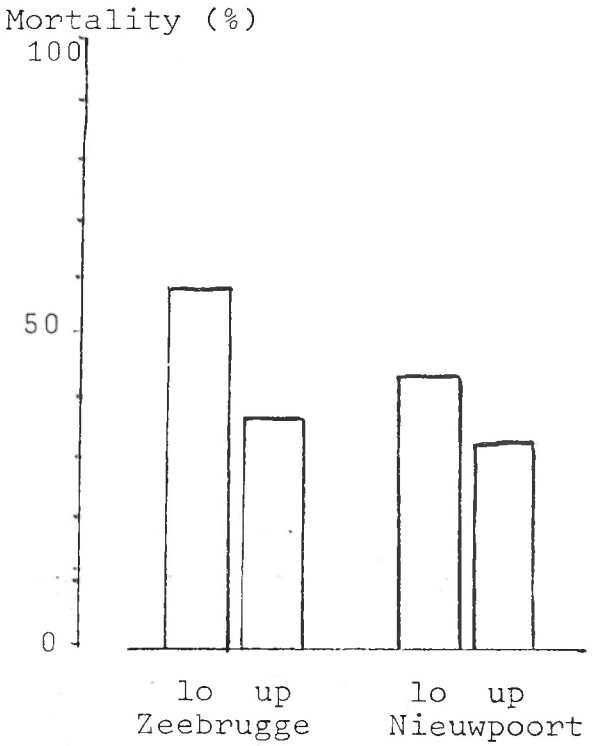


Fig. 3: percentage mortality of the mussels in the cages.

3.1.2. Scope for growth and biometric measurements.

3.1.2.1. Coastal stations.

Table 2 summarizes the physiological measurements on the mussels collected in the four coastal stations. We have used animals of about the same shell size in all four populations (the mean size of the mussels was 48.7 +- 3.2 mm). The results in Table 2 are the real experimental values, uncorrected for differences in dry weight of the mussels. We assumed that the mussels of the same shell length were of about equal age. In doing so, the scope for growth and other physiological measures also reflect the differences in body condition (the degree of filling of the

shells by the soft body parts).

Oxygen consumption is significantly lower in Zeebrugge than in the other stations (t-test, $P < 0.01$). The other three stations do not differ significantly from one another. Presumably, the lower respiration rate in Zeebrugge is a direct reflection of the lower dry weight of the soft body parts.

Table 2.

	Breskens	Zeebrugge	Oostende	Nieuwpoort
R	13.15 (2.82)	8.49 (1.47)	14.71 (4.13)	14.85 (4.93)
E	0.19 (0.12)	0.19 (0.06)	0.66 (0.07)	0.17 (0.09)
CR	3.23 (0.57)	3.44 (0.76)	2.55 (0.48)	3.30 (0.70)
C	23.21 (4.04)	22.79 (5.01)	16.86 (3.19)	23.86 (5.04)
e	91	57	70	93
A	21.14 (3.68)	12.99 (2.85)	11.82 (3.24)	22.29 (4.70)
P	7.79 (3.74)	4.31 (3.51)	-3.95 (4.51)	7.26 (8.14)
GG	32.7 (12.0)	17.1 (11.1)	-21.2 (30.8)	26.0 (28.5)
NG	35.9 (13.2)	30.1 (19.5)	-30.3 (43.9)	27.8 (30.5)
DW	1.221 (0.368)	0.657 (0.101)	1.125 (0.265)	1.454 (0.152)
S/D	44.62 (14.18)	71.72 (10.72)	44.54 (10.31)	35.14 (3.28)

Table 2: Physiological measurements on the mussels from the coastal stations. Mean values (Standard Deviations) of n animals. R: respiration rate (J/h); E: excretion rate (J/h); CR: clearance rate (l/h); C: consumption (J/h); e: absorption efficiency (dimensionless, %); A: Assimilation rate (J/h); P: Scope for growth (J/h); GG: gross growth efficiency (dimensionless, %); NG: net growth efficiency (dimension- less, %); DW: Dry weight of the soft body parts (g); S/D: shell length divided by dry weight (mm/g) providing an index of shell filling.

Ammonia excretion is higher in Oostende than in the other stations (t-test, $P < 0.05$). No significant differences exist between the other three.

Clearance rate and energy consumption are also significantly lower in Oostende (t-test, $P < 0.05$), and do not differ between the other stations.

Absorption efficiency is more than 90 % in Breskens and Nieuwpoort, whereas in Oostende (70 %) and especially in Zeebrugge (57 %) it is considerably lower. For this measurement all animals are pooled in one container, so that no standard deviaton can be given.

Assimilation rates are similar in Breskens and Nieuwpoort; they are significantly higher (t-test, $P < 0.01$) than in the other two stations.

The scope for growth is above 7 J/h in Breskens and Nieuwpoort. It is significantly lower in Zeebrugge (t-test, $P < 0.05$), and has a negative value in Oostende.

Thus we arrive at the following sequence in scope for growth values:

$$O << Z << N < B$$

Gross and net growth efficiency were calculated from the physiological measurements on the individual mussels at each station. They are given by the equations:

$$\text{gross growth efficiency} = K_1 = \frac{A - (R+E)}{C}$$

$$\text{net growth efficiency} = K_2 = \frac{A - (R+E)}{A}$$

The differences in growth efficiency between the stations are very similar to the differences in scope for growth. Only the Zeebrugge values are aberrant, in that they approach the high values found in Breskens and Nieuwpoort.

The dry weight of the soft body is much lower in Zeebrugge than in the three other stations.

The ratio between the length of the shells and the dry weight of the soft body reflects the degree of filling of the shells by the soft body. The higher this ratio, the lower the filling. In Nieuwpoort the mussels are significantly better filled (t-test, $P < 0.01$) than in the other stations. Filling is similar in Breskens and Oostende, where, in turn, the mussels are significantly better filled than in Zeebrugge (t-test, $P < 0.01$).

3.1.2.2. Experimental stations at sea

Table 3 summarizes the physiological measurements on the mussels that were transplanted to the experimental stations. As these mussels come from the same population, the measurements are readily comparable between one another.

The oxygen consumption is significantly different (t-test, $P < 0.05$) between i) Z up and Z low and ii) Z up and N up. No significant difference is found between N up and N lo.

Ammonia excretion is similar between the upper and lower cages in both stations, but it is significantly higher at Zeebrugge than at Nieuwpoort (t-test, $P < 0.01$).

Clearance rate and energy consumption are similar at all stations, except at N up, where they are lower (t-test, $P < 0.05$).

Table 3.

	Zeebrugge		Nieuwpoort	
	Z up	Z lo	N up	N lo
R	11.87 (2.72)	17.84 (7.41)	9.90 (4.85)	9.27 (4.96)
E	0.50 (0.15)	0.42 (0.19)	0.34 (0.12)	0.33 (0.11)
CR	3.13 (1.13)	3.13 (1.20)	2.34 (1.15)	3.24 (1.12)
C	23.16 (9.69)	25.57 (9.56)	16.28 (8.01)	24.85 (8.44)
e	57 %	57 %	93 %	93 %
	78 %	78 %	78 %	78 %
A	13.46 (5.52)	14.57 (5.45)	15.14 (7.45)	23.11 (7.85)
	18.42 (7.56)	19.94 (7.46)	12.70 (6.25)	19.38 (6.58)
P	1.08 (6.20)	-3.68 (8.59)	4.90 (5.21)	13.52 (9.59)
	5.64 (8.48)	1.62 (11.75)	2.42 (4.37)	9.66 (8.04)
GG	-4.8 (19.2)	-25.4 (51.7)	25.3 (37.4)	48.8 (30.4)
NG	8.4 (33.8)	-44.6 (90.6)	27.2 (40.2)	32.5 (32.7)
n	10	9	10	12

Table 3: Physiological measurements on the mussels from the experimental stations at sea. Mean values (Standard deviations) of n animals. R: respiration rate (J/h); E: excretion rate (J/h); CR: clearance rate (l/h); C: consumption (J/h); e: absorption efficiency (dimensionless, %) according to two different assumptions (see text); A: Assimilation rate (J/h); P: Scope for growth (J/h); GG: gross growth rate (dimensionless, %); NG: net growth rate (dimension- less, %). The first row for A and P gives values calcu- lated with absorption efficiencies from the coastal stations; the second row with a constant absorption rate. GG and NG are for the coastal absorption efficiencies.

Due to technical difficulties, we have not been able to estimate the absorption efficiency in any of the four populations. In Table 3 we have given two alternative approximations. First, we have taken the values of absorption efficiency in the corresponding coastal stations. Second, we have taken the mean absorption efficiency of the four coastal stations for the four experimental cages.

Consumption rates are similar in the four cages. It is lower in N up, but not significantly.

Assimilation depends, of course, on the assumption on the absorption efficiency. With the absorption efficiencies of the coastal stations, it is higher in N lo than in the other stations ($P < 0.01$), which do not differ significantly from one another. With a constant absorption efficiency, the assimilation values do not differ significantly between stations.

Taking the absorption efficiency of the coastal stations, the scope for growth is significantly higher at N lo than at N up ($P < 0.05$). It is not significantly different between Z up and Z lo. The sequence of scope for growth is then given by:

$$Z\ lo < Z\ up < N\ up << N\ lo$$

With a constant absorption efficiency, this sequence is somewhat changed. However, the only significant difference still is between N lo and the other stations. We have:

$$Z\ lo < N\ up < Z\ up << N\ lo$$

Table 4 shows the mortality in the experimental mussels. It also includes the biometric data of the population at the onset of the experiment (to) and after the recovery of the cages.

Mortality is lower in the upper cages than in the lower ones. The difference is more pronounced in Zeebrugge than in Nieuwpoort.

The length growth of the shells is higher in Nieuwpoort than in Zeebrugge. The growth values were corrected for the erosion, observed on the empty shells in the cages. All shells were individually measured at the onset of the experiment. The five shells in a cage compartment were either of the same size (to the nearest 0.1 mm) or differed so much in size that they could be recognized after the experiment. Upon recovery we observed that all the empty shells had decreased in length (average decreases were : Z up: -0.305 mm; Z lo : -0.427 ; N up: -0.294 mm ; N lo : -0.361 mm). The decrease is more important in the lower cages, and was interpreted as due to erosion of the fragile shell fringes. Growth was corrected for erosion by assuming that growing shells had suffered the same erosion as the empty ones, and that most mortality had occurred at the onset of the experiment. The latter assumption is reasonable in view of the bad treatment of the animals just prior to the experiment. The growth values thus give the following sequence:

$$Z\ up < Z\ lo < N\ lo < N\ up$$

	Yerseke	Z up	Zlo	N up	N lo
M	-	38 %	60 %	34 %	46 %
G	-	0.26 (0.48)	0.34 (0.40)	0.46 (0.74)	0.45 (0.51)
DW	2.032 (0.415)	1.994 (0.614)	2.034 (0.613)	2.417 (0.486)	2.061 (0.839)
S/D	31.1	33.51 (10.17)	31.91 (8.84)	26.16 (9.01)	32.78 (9.28)
n	9	10	9	10	12

Table 4: Mytilus edulis experiment at sea.
M: Mortality (% of the 50 exposed animals that died during the 52 day experiment); G: growth (mm/52 days) of the surviving animals; DW : dry weight of the soft body parts (g); S/D: shell length divided by dry weight of the soft body (mm/g) providing an index of shell filling. The latter two measurements are based on n animals. All values, except mortality, are means (standard deviation).

3.2. Measurements on the digestive gland.

3.2.1. Analysis of the heavy metals content.

Table 5 shows the cadmium, copper and zinc content of the mussels collected at the coastal stations, purchased at Yerseke and recovered from the experimental stations after 52 days. The heavy metal contents were determined by atomic absorption spectrophotometry using a model 370A Perkin-Elmer flame spectrophotometer after mineralization for 8 h in HNO3 65 % and dilution. They are relatively low and no correlation could be detected between these values and the scope for growth measurements. During the experiment, a slight contamination by cadmium has occurred, whereas the copper and zinc concentrations somewhat decreased. Significance test could not be performed on these data, since all tissues were pooled for the analysis.

Table 5.

	Cd	Cu	Zn
Intertidal populations			
Nieuwpoort	0.3	4.1	20.9
Oostende	0.4	4.0	21.7
Zeebrugge	0.2	3.0	23.2
Breskens	0.7	5.0	31.2
Yerseke	0.0	4.0	30.0
Experimental stations			
N up	0.6	3.2	22.0
N lo	0.3	3.0	23.1
Z up	0.4	3.3	20.8

Table 5: Cadmium, copper and zinc contents (ug/g wet weight) of the digestive glands of mussels from 4 native populations along the Belgian coast, and of the experimental mussels before exposure ("Yerseke"), and after exposure in three of the four cages.

3.2.2. Histological analysis.

The results of the Shikata - test for metalloproteins will not be discussed in detail for the different populations. In all cases the results were very similar. Faintly coloured granules are seen at high magnification. However, these granules are not comparable to the intensily

stained granules present in the reference slides we received from IMER. All the sections should therefore be classified as negative for this test. The problem posed by these faint granules resides in the characteristics of both the test principle and the test animal. M. edulis only starts producing metallothioneins at relatively elevated metal concentrations, which are well above environmental concentrations in the Belgian coastal zone. On the other hand, the test is not specific for metallothioneins. The faintly coloured granules may therefore be interpreted as metalloproteins different from metallothioneins. Moreover, biochemical characterization has been performed at the University of Liege, according to a previously described method (Bouquegneau et al. 1983). No metallothionein was detected (above the background level) by that method either.

3.2.2.1. Coastal stations.

Table 6 shows the mean labilization time of the lysosomal membranes in the four populations, arranged from East to West. Non - parametric comparison in a Friedman multiple comparison test (Sokal & Rohlf, p. XXXX) showed a significant difference between the Nieuwpoort population and the other three ($P < 0.05$). The difference between Zeebrugge and Oostende - Breskens is almost significant ($0.05 < P < 0.10$).

Table 6.

Population	mean (min.)	S.E.	n
Breskens	7.5	1.1	10
Zeebrugge	6.1	0.7	9
Oostende	9.7	1.5	10
Nieuwpoort	18.0	1.3	10

Table 6: Labilisation time (min.) of the lysosomal membranes in four coastal populations of Mytilus edulis. Mean and Standard Error of n observations.

The NTR - test yielded almost no reaction in Nieuwpoort and a faint reaction in Breskens. The reaction was strongly positive in Zeebrugge, but most pronounced in the Oostende population. This gives us the following ordering:

O > Z >> B > N

Due to the stability problems discussed earlier, this ordering can, unfortunately, not be refined any more.

3.2.2.2. Experimental stations at sea.

Table 7 shows the lysosome labilisation time in the Yerseke population (i.e. the mussels at the start of the experiment) and the mussels from the four experimental cages. The labilisation time is generally depressed, but in N up and in Z lo the values are not much lower than in the Yerseke population.

A Friedman multiple comparison test showed that the Yerseke population is significantly different from the others. N up and Z lo are not significantly different from one another, but are significantly higher than N lo and Z up, which in turn are not different. This yields the following sequence:

Y >> N up > Z lo >> Z up > N lo

Table 7.

Cage	mean (min.)	S.E.	n	% degeneration
Z lo	15.0	2.6	9	22
Z up	5.2	0.6	10	47
N lo	4.1	0.5	10	73
N up	18.1	2.6	9	39

Table 7: Labilisation time (min.) of the lysosomal membranes in four experimental stations at sea. Mean and Standard Error of n observations. Also indicated is the mean ratio of tissue degeneration (see text).

The NTR - test shows a distinct pattern: at both stations animals from the upper cages show a much stronger reaction than those of the lower cages, while the reaction is generally stronger off Zeebrugge, than in the station off Nieuwpoort:

Zup > N up > Z lo > N lo

The infestation by M. intestinalis may have had a serious influence on our results. In the literature on M. intestinalis a particular reaction of the mussels to heavy infestation is sometimes described (e.g. Korringa, 1952, Campbell, 1970): the intestinal gland changes in colour from the typical green to very pale, whitish. We found this change back in many (heavily infested) mussels from our experimental cages. Microscopically, the white tissue corresponds to a kind of connective tissue, where the digestive tubuli have completely regressed. The extent to which this apparent degeneration had taken place differed greatly between individuals, but also between cages. In table 7 this is expressed as the ratio of the surface in

the section occupied by the degenerated tissue to the surface occupied by normal tissue, containing tubuli, and degenerated tissue.

4. DISCUSSION.

Table 8 summarizes the results of the different measurements, in the form of an ordering of the stations.

Table 8.

	Coastal Stations	Stations at sea
Mortality	-	Z lo > N lo > Z up > N up
Scope for growth	O << Z << N < B	Z lo < Z up < N up << N lo
Shell growth	-	Z up < Z lo < N lo < N up
metallothioneins	not detected	not detected
NTR	O > Z >> B > N	Z up > N up > Z lo > N lo
Lysosomal stability	Z <= O = B < N	N lo > Z up >> Z lo > N up

Table 8: Summary of the result of the monitoring of 4 coastal stations and 4 experimental cagesat sea. In each class the stations are arranged in increasing or decreasing order of the relevant parameters.

The ordering of the stations is more meaningful than a discussion of the absolute values, especially for the physiological measurements. Many natural parameters may influence these values, and we do not dispose of enough baseline information in our region to classify the measurements as either "high" or "low". As for the histochemical measurements, some form of stress is present in all populations. NTR is higher, and lysosomal stability is lower in all populations than in the Yerseke control population. In contrast, the heavy metal contents can nowhere be described as abnormally high.

It is clear from this table that the stations Oostende and Zeebrugge are less favourable for the mussels than Breskens and Nieuwpoort. Pollution stress in Zeebrugge and Oostende is found back in scope for growth an in NTR. It is not seen in metallothionein contents. Presumably pollution stress along the coast is mostly from organic compounds, and highest near or in the harbours. The lysosomal stability does indicate a form of stress in Breskens which is not reflected in the scope for growth.

Due to stress conditions which act on both variables, SFG and lysosomal stability are often well correlated. However, as is the case in Breskens, they do measure different things. In particular the feeding conditions may provoke different reactions. The Breskens station is

favourable to mussels in several respects. The structure on which the animals were collected protrudes far enough from the sediment to eliminate the eroding influence of sand. Moreover, as the nutrient load of the Scheldt water is high, food conditions may be very favourable in the estuary mouth. We interpret our measurements as an indication of the presence of (pollution) stress in what would otherwise be very good growing conditions.

A priori, we had expected a pllution gradient from the Scheldt river (high pollution) towards the west coast (low pollution). Such a gradient is not apparent from the data. The harbours of Oostende and Zeebrugge clearly had too much influence as (point ?) pollution sources. It would be interesting to study coastal populations in between the harbours, in order to detect i) if the anticipated gradient can be shown, and ii) how far the harbours exert their polluting influence in the coastal waters.

As indicated in the results section, we feel that the infestation with Mytilicola intestinalis might have had a too large influence on the results of our experiment at sea, at least on the lysosomal stability measurements. The data on shell growth, SFG measurements and NTR activity clearly point to a higher pollution influence in the Zeebrugge station. This station is near the Zeebrugge harbour, and in the effluent plume of the Scheldt river. However, the pattern in lysosomal stability might have been clearer without the interfering factor of the M. intestinalis infection.

A problem with this interpretation is that the pathological conditions described are in accordance with older observations, but contrast with some recent assessments of the effects of M. intestinalis on mussels (e.g. Dare, 1985). Moore et al. (1977) did not find any histological changes in Mytilus edulis as a response to Mytilicola intestinalis, except for a limited damage to the gut epithelium at the places where M. intestinalis is in direct contact with it (here the cilia are reduced or absent). Several additional studies have shown that M. intestinalis usually does not have serious deleterious effect on Mytilus edulis (Davey et al., 1978; Gee et al., 1977; Bayne et al., 1977). The only exceptions are when high infestation levels coincide with other stress factors. This led Dare (1985) to the conclusion that Mytilicola intestinalis should be considered as a relatively harmless commensal, rather than a parasite of Mytilus edulis. In view of these studies, our conclusion that Mytilicola intestinalis might be responsible for the apparent tissue degeneration must be viewed with some caution. It was based on the reports of similar observations, and on the absence af any other apparent causal agent. This subject surely needs further (experimental) research. Anyway, it seems better to perform similar experiments in the future with Mytilicola-free mussels.

A factor of unknown importance in this study is the quality and quantitiy of the food available to the mussels. This factor is not only important for the measurements performed here, but has considerable importance in its own, in view of the eutrophication problems in the Belgian

coastal zone. In our experiment the different reactions of scope for growth on the one hand, and NTR on the other hand could be due to this factor. Eutrophication may have a positive effect on scope for growth, whereas it may not induce a particular reaction in the NTR experiments. It is an advantage of the integrated monitoring strategy that the different measurements allow one at least to hypothesize on this kind of mechanisms.

The difference in NTR - reaction between the upper and lower cages is remarkable in these shallow stations. After all, the cages were only a few meter apart. The shell growth and erosion data also indicate differences in the conditions between the upper and lower cages. In deeper water (40 - 50 m) Johnson et al. (1984) also found a marked difference between upper and lower cages in NTR - activity. However, this difference was at least as large in their one shallow station (< 10 m) ! The substances that provoke the NTR - reaction have a low specific weight (e.g. aromatic hydrocarbons). The fact that they exert more influence at lower depths would indicate that their concentration is higher near the surface. This could be the case if they are not completely in dissolved form, or if by some mechanism they are skimmed out of the water and taken to the surface (e.g. by adsorption to rising air bubbles). If this tentative conclusion could be confirmed then even shallow, well-mixed coastal waters should explicitly be considered as three-dimensional for any measurement of pollution concentration. It would be interesting to study if vertical concentration gradients could directly be found for organic pollutants.

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