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Ecophysiological study of the Pacific oyster *Crassostrea gigas* naturally infected by a *Chlamydia*-like microorganism: effect of infection level and diet on oyster physiological responses

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RÉSUMÉ

Etude écophysiologique de l'huître japonaise *Crassostrea gigas* infectée naturellement par un microorganisme de type *Chlamydia*: effets du niveau d'infection et du régime alimentaire sur la réponse physiologique de l'huître.

Une population d'huîtres japonaises, *Crassostrea gigas*, infectée par un procaryote de type *Chlamydia* a été élevée pour comparer l'évolution de l'infection sous trois régimes alimentaires: le jeûne, un régime intermédiaire à base de *Skeletonema costatum*, et un régime mixte de microalgues. Plusieurs critères physiologiques et pathologiques: présence d'anomalies de la branchie; examen microscopique, inclusions intracytoplasmiques basophiles, réaction de Feulgen; indice de condition, filtration, consommation de nourriture; production de fèces et respiration ont été utilisés pour comparer les différentes modalités trois mois après le début de l'expérience. Les huîtres maintenues à jeun présentent des performances physiologiques inférieures en termes de croissance et d'indice de condition, comparées à celles nourries avec *Skeletonema costatutm*, ou avec le mélange de *Isochrysis galbana* (souche Tahitienne), *Tetraselmis suecica*, *Chaetoceros calcitrans* et *Pavlova lutherii*.

Un indice de condition de 7,4 reflétait un stade de maturation avancée dans la population nourrie avec le mélange de microalgues. Quoique infectées par un microorganisme de type *Chlamydia* les deux populations nourries avaient des réponses physiologiques remarquables (bons indices de condition et potentiels de croissance positifs).

Le potentiel de croissance a été comparé à la production effective en tenant compte de la maturation et du stade de pré-ponte des huîtres. Une corrélation positive a été établie entre les anomalies branchiales et le taux de filtration pour les huîtres à jeun et celles nourries avec *Skeletomema*. Dans les populations infectées par le microorganisme de type *Chlamydia*, les estimations de filtration sont un meilleur indicateur pour les taux d'infection élevés, que les mesures physiologiques effectuées (respiration, production de fèces par exemple) ou estimées (taux d'absorption, potentiel de croissance) physiologiques. En conséquence, la relation entre critères pathogènes et mesures physiologiques peut être utilisée pour une estimation de l'infestation. Toutefois, une identification spécifique des agents pathogènes est nécessaire, dans une étape suivante, pour une estimation quantitative des effets pathogènes.

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ABSTRACT

A population of Pacific oysters, *Crassostrea gigas*, infected with a *Chlamydia*-like procaryote was reared to compare infection trends under three different feeding conditions: a starved condition, an intermediate condition with a diet of *Skeletonema costatum*, and a mixed algal diet. Several physiological and pathological criteria: presence of gill abnormality; microscopic examination, basophilic intracytoplasmic inclusions, Feulgen reaction; condition index, filtration, food consumption; fecal production and respiration were used to compare the various modalities one month after the initiation of the experiment. The modality of starved oysters showed reduce physiological performances in terms of scope for growth and condition index compared to oysters fed *Skeletonema costatutm*, or a mixed diet of *Isochrysis galbana* (Tahitien strain), *Tetraselmis suecica*, *Chaetoceros calcitrans* and *Pavlova lutherii*.

A condition index of 7.4 reflected a prespawning stage in the population fed with a diet of mixed algae. Although infected with a *Chlamydia*-like microorganism, impressive physiological responses (good condition index and positive scope for growths) were reported for both fed oyster populations. The scope for growth values compared to effective production were discussed with regard to the maturation process and the pre-spawning stage of the oysters.

A positive linear relationship was established between gill abnormalities and filtration rate for starved and *Skeletonema* fed oysters. In populations known to be infected with the *Chlamydia* - like organism examined in this study, clearance rate estimates represent a better bioindicator for high infection level than compared to either physiological measurements (e.g., respiration, feces production) or estimates (e.g., absorption rate, scope for growth). Thus, the relationship between pathological criteria and physiological measurements can be used to estimate pathogen diagnosis. However, specific pathogen identifications should be carried out as a further step to quantitatively assess pathogen effects.

INTRODUCTION

Mortalities resulting from the effect of prokaryotic parasites have been reported in many species of bivalve: *Donax trunculus* by Comps and Raimbault (1978), *Tellina tenuis* by Buchanan (1978), *Placopecten magellanicus* by Gulka *et al.* (1983), *Siliqua patula* by Elston (1986), *Pecten maximus* by Le Gall *et al.*, (1988). However, the relationship between parasite infection and its effect on physiological functions have rarely been described (Newell; 1985; Le Gall *et al.*,1991). Newell (1985) reported a significant reduction in feeding rate of *Crassostrea virginica* infected with the MSX parasite, *Haplosporidium nelsoni*. Moreover, Le Gall *et al.*,(1991), pointed out the energetic loss due to rickettsia infection in *Pecten maximus*.

This study was designed to test the capacity of oyster *Crassostrea gigas* to recover from a *Chlamydia*-like organism infection. Three oyster groups were each fed a different diet. The experimental oyster population, originated from the Marennes-Oléron Bay (France), was naturally infected with a *Chlamydia*-like organism. Infection recovery was evaluated using whole animal physiological measurements and gross and histo pathological examination of gills and mantle tissues.

MATERIALS AND METHODS

Experimental set up

Two year old oysters were collected from 'Le Chapus' in the mid-part of Marennes-Oléron Bay, divided into three groups and reared in a closed raceway system. No food was provided to the first group (ST). Two additional groups were fed *ad libitum*. Oysters from the second group (DS) were fed *Skeletonema costatum* four times a day cultured in 300 m^3 tanks. A mixed diet (DM) of cultured algae including *Isochrysis galbana* (Tahitien strain), *Tetraselmis suecica*, *Chaetoceros calcitrans* and *Pavlova lutheri* was provided to the third oyster batch. The closed system is based on a continuous seawater circulation through biological filters, which induced a 1 mg ml^{-1} organic matter production in the raceways. Although limited, the food distribution required a regular seawater input into the raceways. Temperature ranged from 14°C to 20°C, with a 16.4°C average during the feeding management lasting from April 24 to May 25. The experimental setup was described in detail by Barillé *et al.*, (1993).

Sea water and oyster biodeposits sampling

The experimental set up included 12 individual chambers. Ten chambers were used with oysters for physiological measurements and 2 chambers, without oysters as control to estimate inflow seawater characteristics (POMe) (figure 1). Seawater temperature was constant during the physiological measurements (15°C). Hydrological measurements were estimated in triplicate. Total particulate matter (TPM) was estimated by filtering seawater on Whatman GF/C filters and then dried at 60°C before weighing. Particulate organic (POM) and inorganic matter (PIM) were estimated by weighing after ignition at 450°C (Razet *et al.*, 1990). The seawater characteristics remained constant throughout the experiments. Mean seston load was maintained below the pseudofeces production threshold (4.6 mg l^{-1}) (Deslous-Paoli *et al.*, 1992).

Each oyster was glued on a division to facilitate feces and pseudofeces sampling (figure 1). Biodeposits were collected every 2 hours. Feces production were delayed by considering the gut transit time (35 min) (Soletchnik *et al.*, 1996).

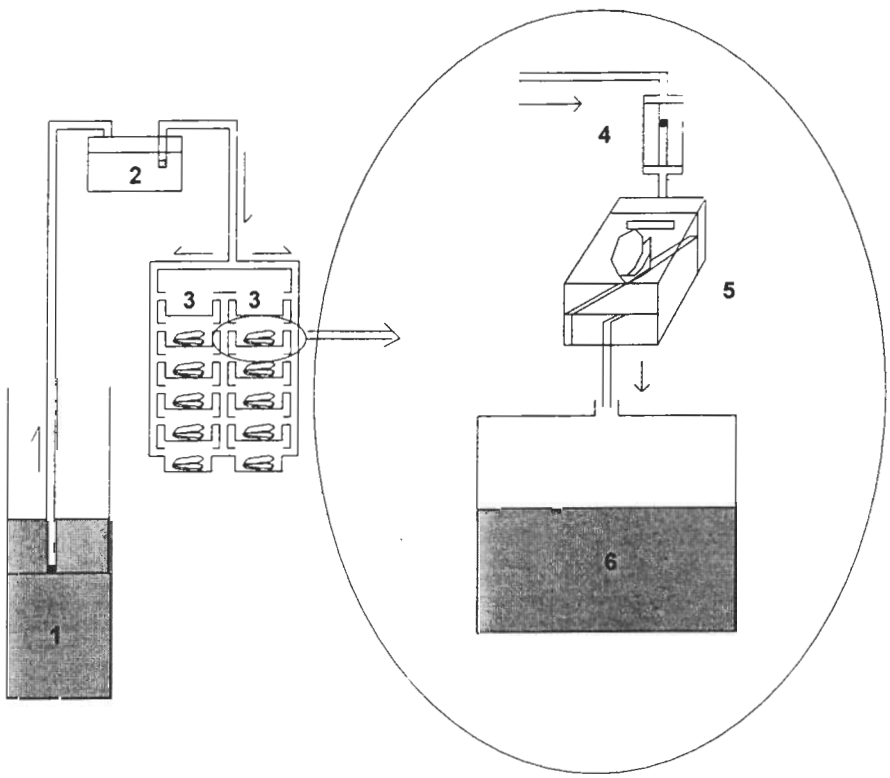


Figure 1. Experimental setup: 1, Algal (*Tetraselmis suecica)* reservoir; 2, Mixing tank; 3, set of measurement chamber; 4, flowmeter; 5, detail of a measurement chamber with oyster; 6 outflowing water collect tank.

Oyster biometry and proximate biochemical composition

Once the experiment was completed, the oysters were shucked. After freezing and then freeze-drying for 36 hours, meat were weighed (DMW) individually to the nearest 0.01 mg. Dry shell (DSW) was weighted to the nearest 0.1 g. Proximate biochemical analyses were then performed on the dry oyster meat. Carbohydrates (CARB) and glycogen (GLYC) concentrations were analyzed using the method of Dubois *et al.*, (1956), proteins (PROT), using the Lowry *et al.*, (1951) method. After extraction (Bligh and Dyer, 1959), lipids (LIPI) were analyzed using the method of Marsh and Weinstein (1966).

Physiological functions

Physiological measurements as well as further pathological analysis were carried out individually on 24 oysters per treatment (ST, DS, DM). Clearance rates were estimated using the total sea water volume flowing through the experimental set-up to allow precise particulate matter concentration estimates (figure 1). Respiration rates were estimated using oxymetric Orbisphere probes (Orbisphere laboratories). The Walne and Mann (1975) condition index (CONDI) was calculated as:

$$CONDI = 100 \,.\, DMW \,/\, DSW.$$

Consumption rate (mg h^{-1}) (CONSU) was calculated using :

$$CONSU = Vol \,.\, (POM_i - POM_o)/\, t \,.\, 60$$

where, Vol represented the total volume of seawater collected during the experimental time; POM was the concentration of organic matter in control' seawater (inflow) (POM_i) and experimental chambers' outflow (POM_o), respectively.

Physiological estimates (i.e., respiration and clearance rates) were standardized to dry meat weight using the following allometric relationship (Bayne and Newell, 1983):

$$V_{std} = (DMW_{std}/DMW_{exp})^b \times V_{exp}$$

V_{exp}, measured clearance (CLEAR) or respiration (RESPI) rate was standardized (V_{std}) to a 1g dry meat weight oyster (DMW_{std}). The allometric coefficient b was 0.8 and 0.439 for respiration and clearance rates, respectively. Respiration and clearance rates were fitted to models developed by Bougrier (Bougrier *et al.*, 1995). These two models are temperature (TEMP) and weight (DMW) dependant:

$$CLEAR = [4.825 - (0.013 \times (TEMP-18.954)^2)] \times DMW^{0.439}$$

$$RESPI = [-0.432 + (0.613 \times 1.042^{TEMP})] \times DMW^{0.8}$$

Since no pseudofeces were produced, consumption rate was considered similar to ingestion rate. Therefore, the organic absorption A (mg $h^{-1}g^{-1}$) was calculated by subtracting the organic portion of the feces F (mg $h^{-1}g^{-1}$) from the POM consumed.

Absorption rate (%) was the A/C ratio calculated from Winberg (1957).

Energetic budget

The scope for growth (SFG) was estimated as follows:

$$SFG = A - RESPI$$

The energy balance was calculated using POM from *Tetraselmis* sp in natural filtered seawater. This represents the 'standard water' for physiological measurements (Widdows, 1985).

Energetic conversion factors were 17 J mg^{-1} and 0.45 J $\mu mole^{-1}$ for *Tetraselmis suecica* and oxygen respectively (Romberger and Epifanio, 1981; Whyte, 1987). Shell energy was equivalent to 0.21 J mg^{-1} (Dame, 1972) and dry meat weight to 20.18 J mg^{-1} (Héral and Deslous-Paoli, 1983). Moreover, Deslous-Paoli and Héral (1980) estimated the gonad conversion factor to 20.39 J mg^{-1}

Pathology

From the initial stock of oysters, the occurrence of basophilic irregular intracytoplasmic inclusion bodies, in gill and mantle connective tissue, was 59% in March 1993.

After completion of physiological measurements, gross examination and pathological sampling for histological analyses were systematically carried out on the oysters.

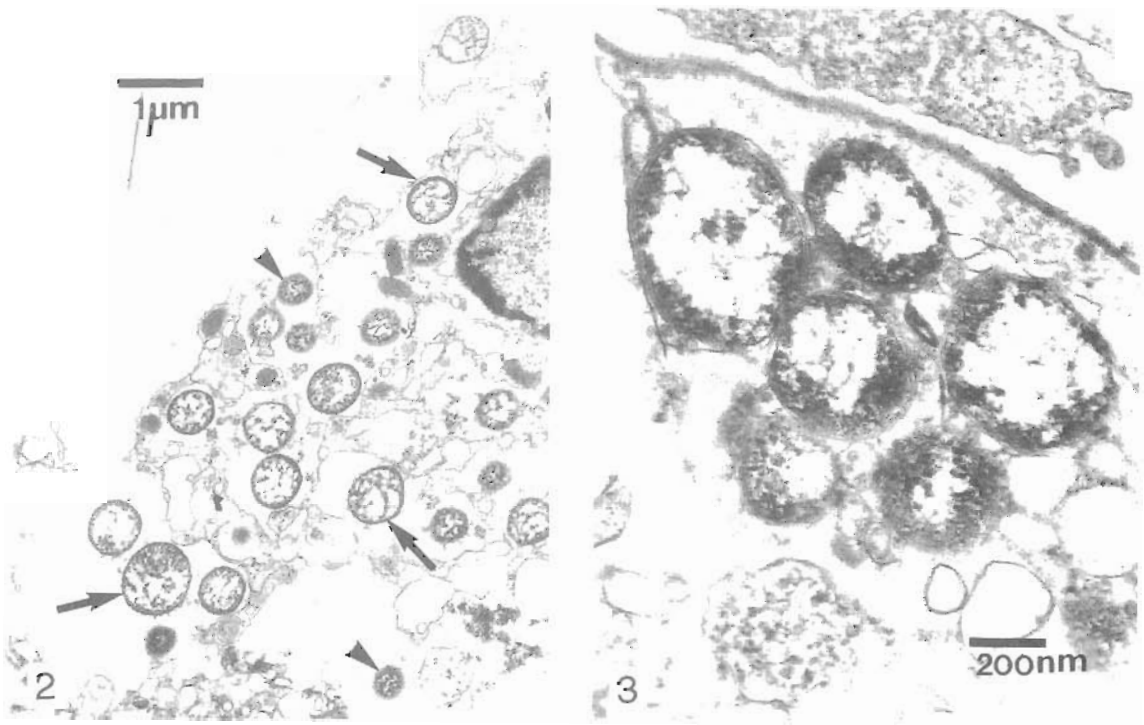
Gills and mantle samples tissues were fixed using Davidson's fluid, then embedded in paraffin for further histological examination. The 3 nm tissue sections were stained by Hematoxylin-Eosin or by nucleal Feulgen reaction, then checked for lesions using a photomicroscope.

Five different types of abnormalities were then detected. The first one was described by gross examination and the others using histological analysis.

The variable (MACRO) represented abnormalities detected by gross examination. Paraffin sections stained in Hematoxylin and eosin or by nucleal Feulgen reaction showed four different abnormalities:

- Mass hemocytic cellular infiltration in connective tissues of several organs (HEMIN).
- Drastic changes of the normal gill structure in several areas like filament fusion (GABNO).
- Irregular, basophilic cytoplasmic inclusion bodies in connective tissue of gills and mantle (BASIN).
- Irregular Feulgen positive cytoplasmic inclusion bodies in connective tissue of gills and mantle (FEULG).

Moreover, the *Chlamydia*-like infection was individually confirmed by transmission electronic microscopic analysis (TEM) (figures 2 and 3).



Figures 2-3. Transmission electron micrographs of *Crassostrea gigas* gill tissues. 2: A gill cell infected with *Chlamydia* - like organism: reticulate bodies (arrows) and intermediate condensing (arrowheads). *Bar: 1 µm.* 3: Transmission electron micrograph of *Crassostrea gigas* gill tissues. Details of reticulate bodies. *Bar: 200 nm.*

Statistical analysis

Physiological responses were compared using Chi squared tests (Schwartz, 1963). A correlation matrix was calculated using the following variables:

- pathological variables: basophilic inclusions (BASIN), nucleal Feulgen reaction (FEULG), gill abnormalities detected by using both photomicroscope (GABNO), and macroscopical observation (MACRO).

- biometric variables: dry meat weight (DMW, g), dry shell weight (DSW, g), condition index (CONDI).

- physiological variables: respiration (RESPI, mg O_2 h^{-1}), clearance rate (CLEAR, l^{-1}), consumption rate (CONSU, mg POM h^{-1}), feces production (FECES, mg h^{-1}).

Variance of the main biological components was calculated to compare feeding treatments (ANOVA). The dry meat weight (DMW) (or the dry shell weight DSW) was as a covariable.

Then, a multivariate analysis, (Principal Component Analysis, PCA), based on the previous correlation matrix was performed to consider simultaneously physiological and pathological data. The main objective was to assess the contribution of each variable in terms of variabilities as to estimate relationships among descriptors. The second objective was to discriminate oyster groups based upon physiological and pathological criteria.

RESULTS

Oyster biometry and proximate biochemical composition

One characteristic of the oyster population resulting from the DM diet was an increased shell growth and sexual maturity for almost 90% of the individuals. Condition index of the DM group was three times greater than starved oysters (ST). Dry meat weight and condition indices were 0.59, 0.91, 1.93 g and 2.74 %, 4.36 %, 7.41 %, respectively for the groups ST, DS and DM (table 1).

Table 1. Biochemical composition of the three groups of oysters after one month feeding on different diets. ST: starvation condition; DS: *Skeletonema costatum*; DM: mixed algal diet. (±CL): ± 0.05 confidence limits.

Diets	ST (±CL)	DS (±CL)	DM (±CL)
dry meat weight (g)	0.59 (±0.09)	0.91 (±0.11)	1.93 (±0.17)
condition index	2.74 (±0.38)	4.36 (±0.31)	7.41 (±0.62)
proteins (%)	28.24 (±2.75)	25.27 (±2.09)	29.44 (±3.82)
lipids (%)	8.48 (±0.97)	9.63 (±1.13)	12.37 (±1.66)
carbohydrates (%)	4.15 (±1.31)	5.92 (±0.96)	3.39 (±0.47)
glycogen (%)	1.77 (±0.92)	2.11 (±0.77)	0.57 (±0.29)

A covariance analysis (Ancova) was performed for the main biochemical components: PROT, LIPI, CARB and GLYC. Significant differences between the ST, DS and DM groups, were observed for all components ($p = 0.05$ for GLYC and $p = 0.001$ for the others components). The covariable was the dry shell weight (table 2; figure 4). Results were more significant when the dry meat weight (DMW) was considered as a covariable (table 3; figure 5). In the latter case, no significant differences were observed among groups with PROT and LIPI, but the CARB and GLYC at 1% and 5% threshold level respectively, reflected the use of carbohydrate during the maturation processes of the DM oyster group. The covariable effect (DMW or DSW) was significant for each biochemical compound.

Table 2. Covariance analysis of the various biochemical analysis (variables: PROT: protein, LIPI: lipid, CARB: carbohydrate, GLYC: glycogen) of oysters dry meat for the three treatments (ST: starvation condition; DS: *Skeletonema costatum;* DM: mixed algal diet). The covariable is the dry shell weight (DSW).

	Sum of Squares	d.f.	Mean square	F. ratio	Sig. level
COV [DSW]	0.319	1	0.319	12.45	0.0008
V [PROT]	1.375	2	0.687	26.84	0.0000
residual	1.716	67	0.025		
total	4.233	70			
COV[DSW]	0.074	1	0.074	15.64	0.0002
V[LIPI]	0.281	2	0.140	29.39	0.0000
residual	0.320	67	0.004		
total	0.849	70			
COV [DSW]	0.005	1	0.006	8.59	0.0046
V [CARB]	0.013	2	0.006	10.02	0.0002
residual	0.044	67	0.0006		
total	0.067	70			
COV [DSW]	0.0014	1	0.0014	5.60	0.021
V [GLYC]	0.0018	2	0.0009	3.60	0.032
residual	0.0173	67	0.0003		
total	0.0199	70			

Table 3. Covariance (COV) analysis of the various biochemical analysis (variables: PROT: protein, LIPI: lipid, CARB: carbohydrate, GLYC: glycogen) of oysters dry meat for the three treatments (ST: starvation condition; DS: diet on *Skeletonema costatum;* DM: mixed algal diet). The covariable is the dry meat weight (DMW).

	Sum of Squares	d.f.	Mean square	F. ratio	Sig. level
COV [DMW]	1.136	1	1.135	84.80	0.000
V [PROT]	0.030	2	0.015	1.21	0.331
residual	0.898	67	0.013		
total	4.233	70			
COV[DMW]	0.244	1	0.244	109.3	0.000
V[LIPI]	0.004	2	0.002	0.89	0.413
residual	0.150	67	0.002		
total	0.849	70			
COV [DMW]	0.017	1	0.017	36.9	0.000
V [CARB]	0.007	2	0.003	7.57	0.001
residual	0.031	67	0.0004		
total	0.067	70			
COV [DMW]	0.0014	1	0.0014	5.44	0.022
V [GLYC]	0.0024	2	0.0012	4.72	0.012
residual	0.0174	67	0.0002		
total	0.0200	70			

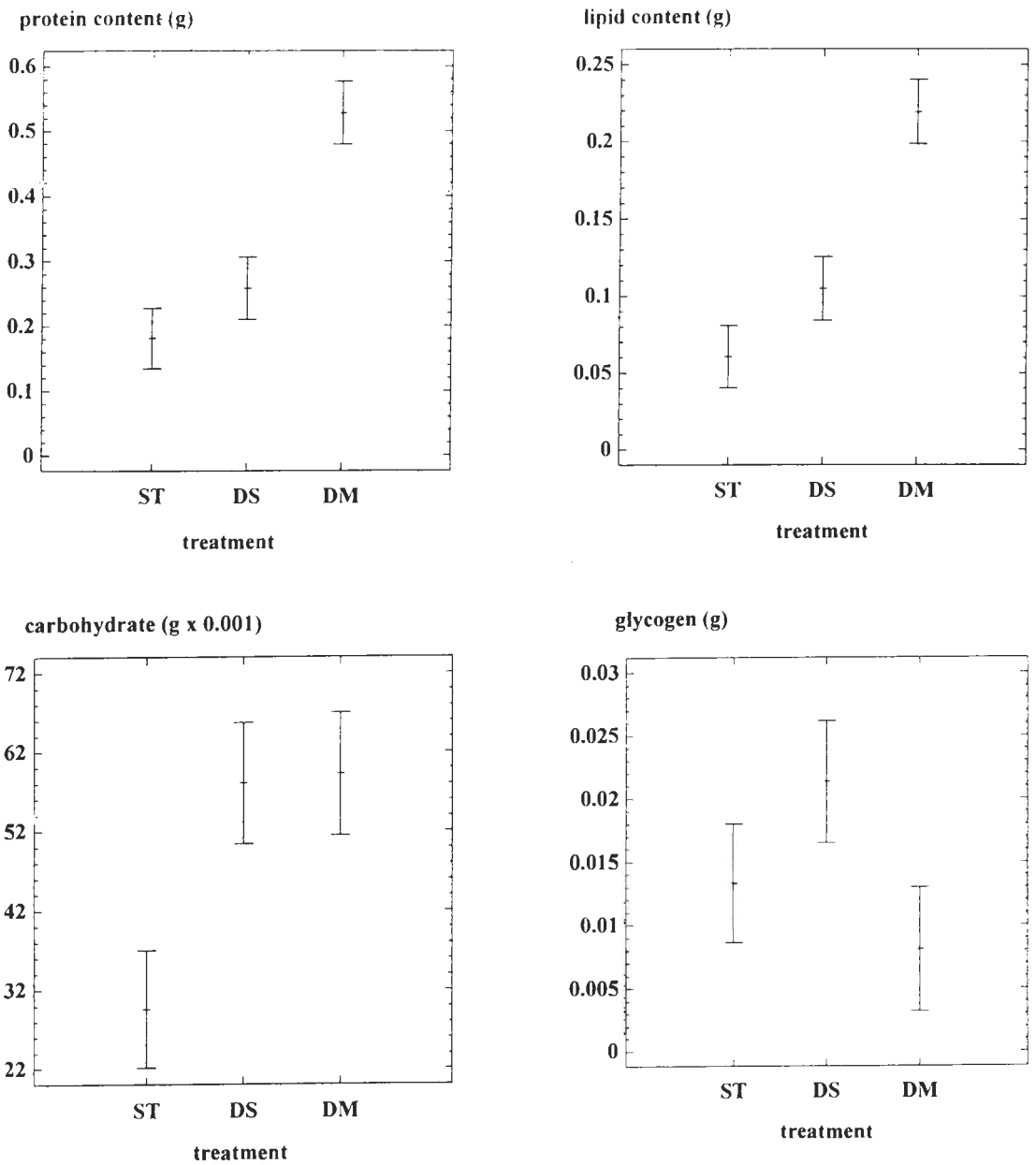


Figure 4. Mean and confidence limits of the biochemical components of oysters dry meat (protein, lipid, carbohydrate, glycogen) for the three treatments (ST: starvation condition; DS: *Skeletonema costatum;* DM: mixed algal diet). The covariable is the dry shell weight.

Physiological responses

The covariance analysis of clearance and respiration showed no significant differences among the groups of oysters fed the three diets (ST, DS, DM) (table 4, figure 6). The respiration rates were significantly correlated to the DMW covariable.

Total particulate matter concentration was 4.09 mg l^{-1}, below the pseudofeces production threshold. Organic matter content reached 73%. The average 3.19 $l^{-1}g^{-1}$ clearance rate was lower for DM treatment compared to others. In contrast, organic consumption rate (5.75 mg h^{-1} g^{-1}) for DS treatment was significantly higher than for the others. The 0.39 and 0.40 absorption rates were similar for DS and DM conditions.

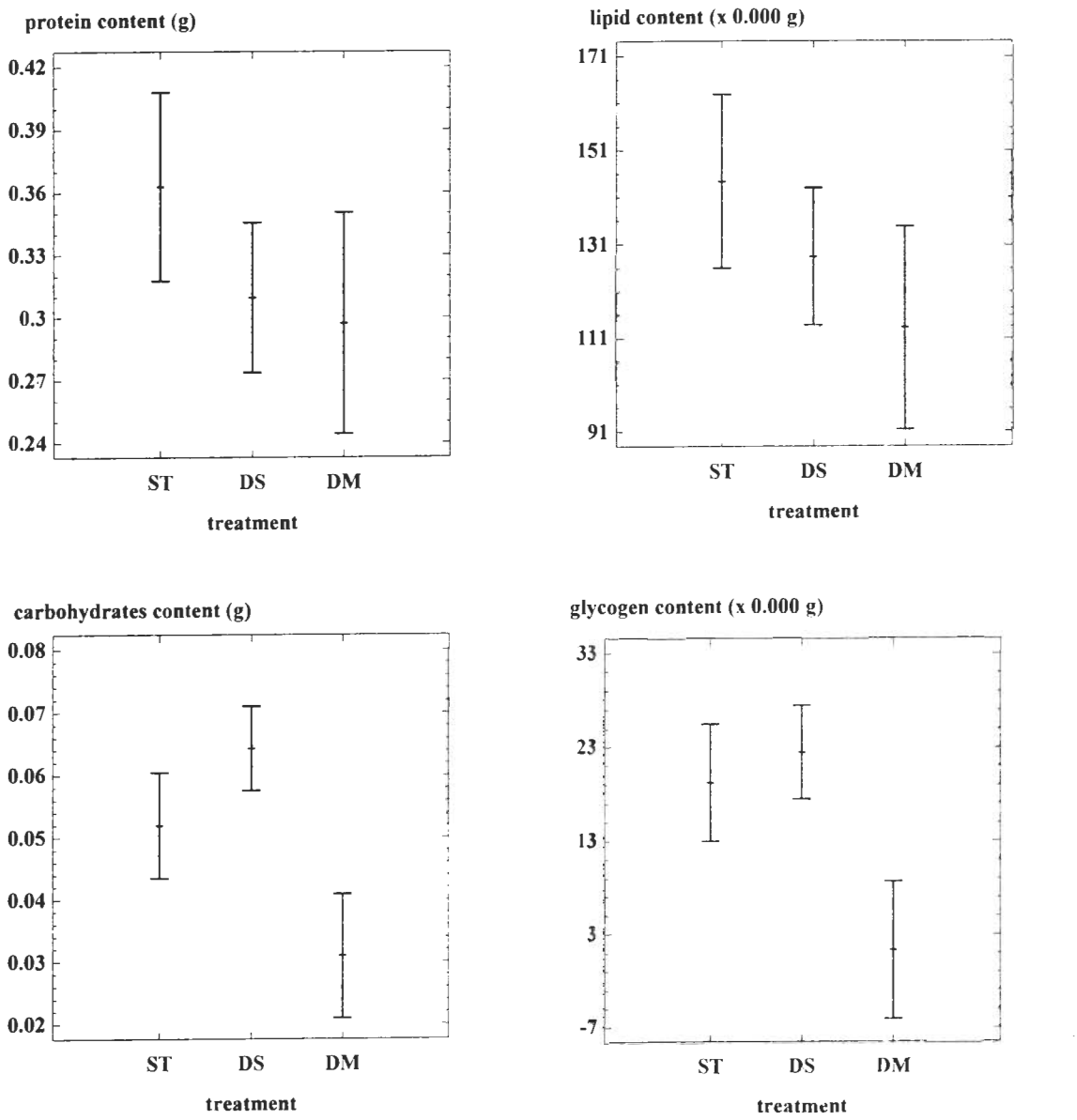


Figure 5. Mean and confidence limits of the biochemical components of oysters dry meat (protein, lipid, carbohydrate, glycogen) for the three treatments (ST: starvation condition; DS: *Skeletonema costatum;* DM: mixed algal diet). The covariable is the dry meat weight.

Organic matter absorption reached 2.22 mg h^{-1} g^{-1} and 1.19 mg h^{-1} g^{-1} for treatment DS and DM, respectively. A negative absorption rate reflected the reduced oyster physiological condition in treatment ST. The standard metabolism, ranged from 10-13 J h^{-1} g^{-1}, was not different among the three treatments (table 3). While scope for growth was negative in treatment ST, it reached 25 and 10 J h^{-1} $g^{-1}$for treatment DS and DM, respectively, without statistical significant differences. Moreover, the -22 J h^{-1} g^{-1} negative scope for growth demonstrated the poor condition of oysters subject to ST treatment.

Table 4. Covariance analysis of the clearance rate (CLEAR) and respiration (RESPI) of oysters for the three treatments (ST: starvation condition; DS: diet on *Skeletonema costatum*; DM: mixed algal diet). COV: covariance; V: variance. The covariable (COV) is the dry meat weight (DMW).

	Sum of Squares	d.f.	Mean square	F. ratio	Sig. level
COV [DMW]	0.115	1	0.115	0.100	0.75
V [FILT]	0.225	2	0.113	0.098	0.91
residual	68.08	59	1.15		
total	68.44	62			
COV[DMW]	0.844	1	0.844	10.33	0.002
V[RESPI]	0.208	2	0.104	1.27	0.287
residual	4.820	59	0.081		
total	11.40	62			

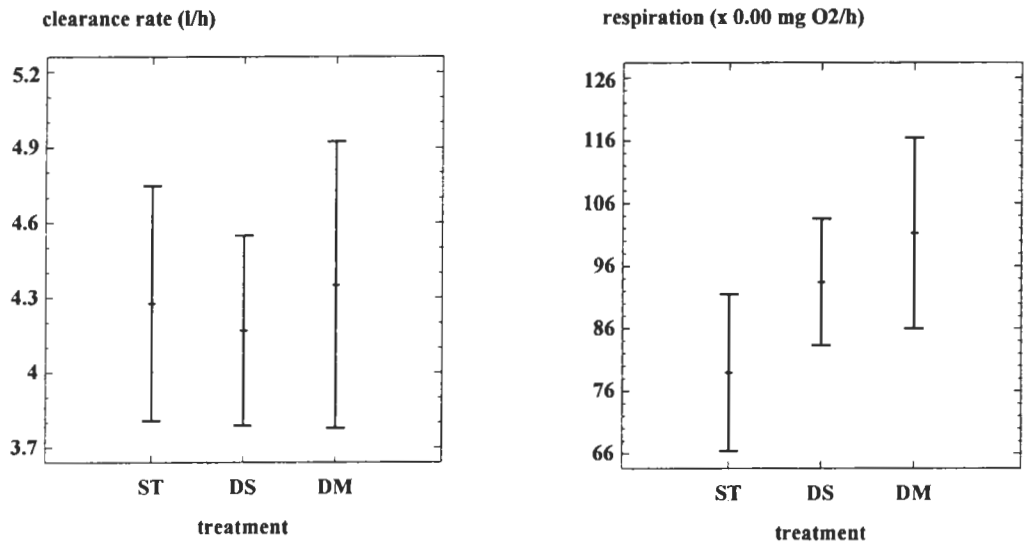


Figure 6. Mean and confidence limits of the clearance rate and respiration of oysters for the three treatments (ST: starvation condition; DS: *Skeletonema costatum*; DM: mixed algal diet). The covariable (COV) is the dry meat weight (DMW).

Comparisons between model outputs and our clearance and respiration rate measurements are reported in figures. 7 and 8. Three groups were discriminated: ST and DS measurements were well fitted to the respiration model. In contrast, most of the measured respiration rates for treatment DM were below the calculated values (figure 7). With regards to clearance rate measurements, three groups were observed along an horizontal axis (figure 8). However, most of the oyster responses from the group ST were correctly fitted to the model. A group of oysters was characterized by a significantly higher clearance rate than model estimates. Moreover 80% of these oysters were characterized by gill abnormalities under histological observations (GABNO) and included half of the oysters showing this pathological criterion.

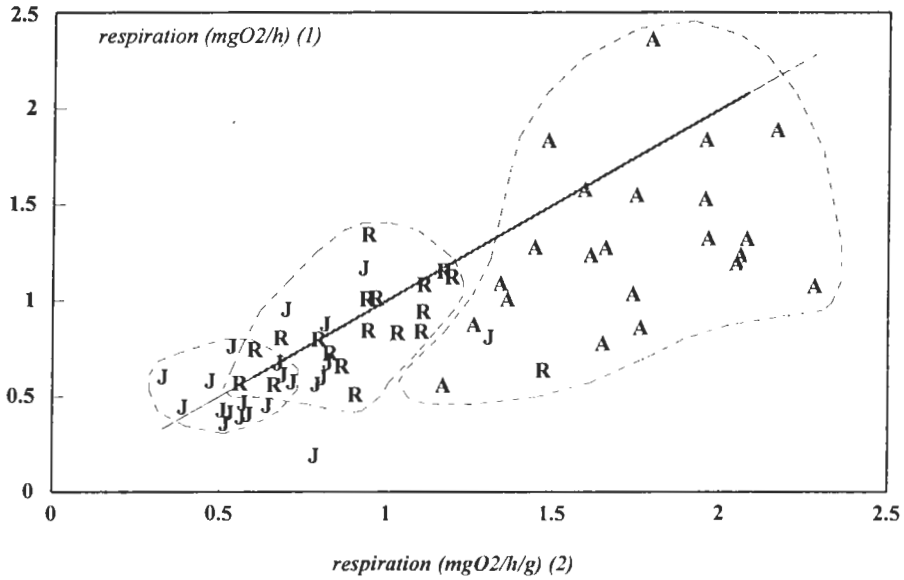


Figure 7. Oyster experimental respiration results (1), fitted to the respiration model (2): RESPI = [-0,432 + (0,613 x $1{,}042^{TEMP}$)] x $DMW^{0,8}$ (Bougrier et al., 1995). J=ST: starvation condition; R=DS: *Skeletonema costatum;* A=DM: mixed algal diet.

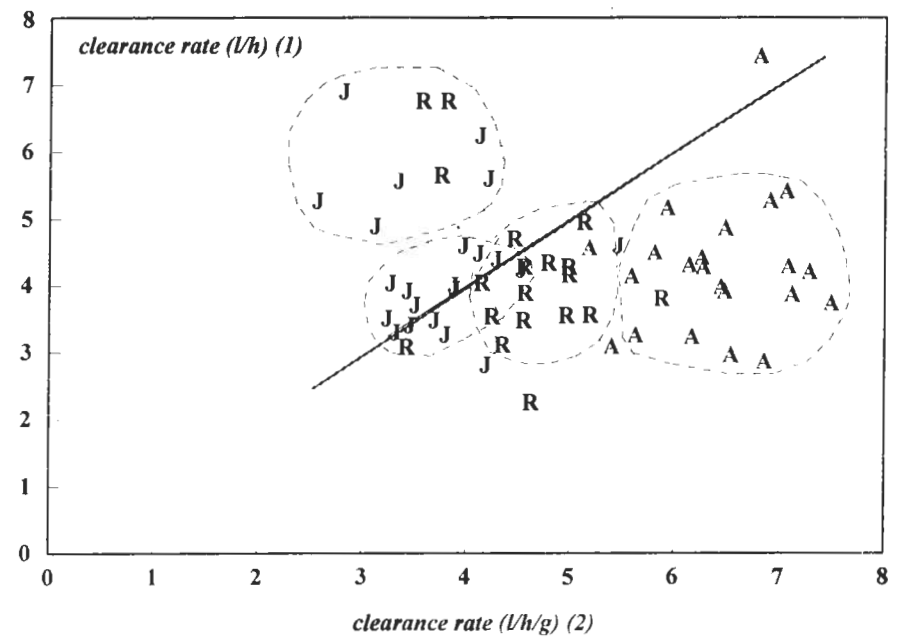


Figure 8. Oyster experimental clearance rate result (1), fitted to the clearance rate model (2): CLEAR = [4,825 - (0,013 x $(TEMP-18{,}954)^2$)] x $DMW^{0,439}$ (Bougrier et al., 1995) J=ST: starvation condition; R=DS: *Skeletonema costatum;* A=DM: mixed algal diet.

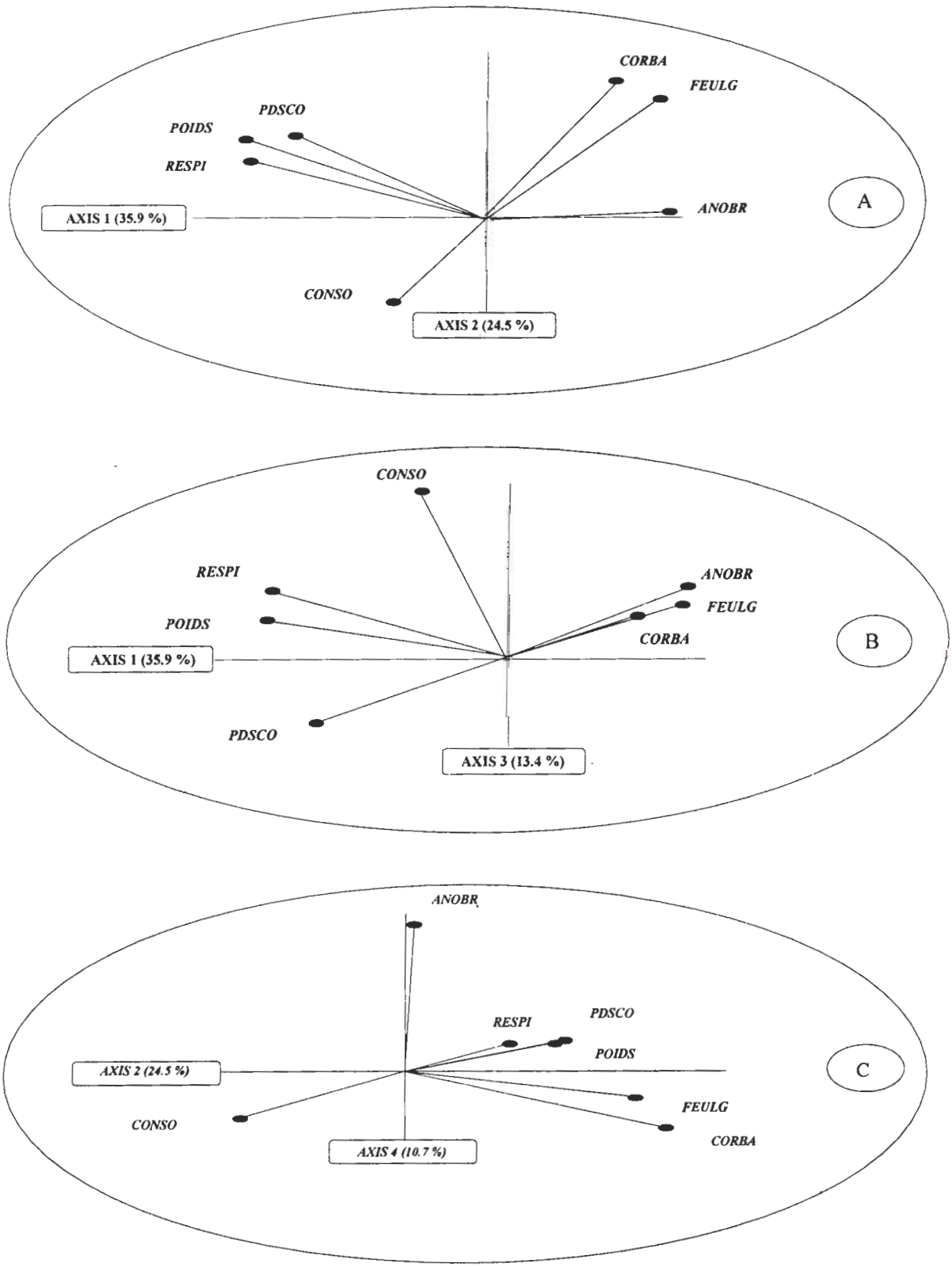


Figure 9. Correlation between descriptors and principal axes in a Principal Componant Analysis (PCA). Variable contribution on 1, 2 3 and 4 axis.

Table 5. Main Physiological responses and Energetic budget based on organic matter. ST: starvation condition; DS: *Skeletonema costatum;* DM: mixed algal diet. Results are standardized to 1 g dry meat weight oyster according to Bayne and Newell (1983). (±CL): ± 0.05 confidence limits.

Measurements	Diets ST (±CL)	DS (±CL)	DM (±CL)
clearance rate (liter h^{-1})	5.87 (±0.84)	4.59 (±0.72)	3.19 (±0.31)
consumption (mg h^{-1})	3.64 (±1.23)	5.75 (±0.91)	2.93 (±0.67)
absorption (mg h^{-1})	-0.59 (±0.91)	2.22 (±0.61)	1.19 (±0.43)
absorption efficiency (%)	-0.17 (±0.35)	0.39 (±0.09)	0.40 (±0.10)
absorption (J h^{-1})	-9.97 (±15.41)	36.04 (±10.45)	20.20 (±7.43)
respiration (J h^{-1})	12.60 (±2.30)	11.43 (±2.36)	10.36 (±1.65)
scope for growth (J h^{-1})	-22.57 (±15.91)	25.09 (±10.70)	9.84 (±7.50)

Pathology

Several oysters had microscopically visible gill lesions that appeared as indentations. Most of the affected oysters showed one to several indentations on one or several lamellae. When oysters were heavily infected with the *Chlamydia*-like organism using TEM's analysis, the whole lamella was affected and appeared unusually thin. No significant difference in macroscopical lesions were observed in infected individuals in the three treatments ST (66.6 %), DS (58 %) and DM (50 %) ($p > 0.05$, Chi-square test).

A Chi-squared test was performed on the histologicaly detectable abnormalities associated with rearing conditions (table 6). No significant difference was observed among treatments ST, DS and DM, for either basophilic intracytoplasmic corpuscles (BASIN), Feulgen positive inclusion bodies (FEULG), or mass hemocytic infiltration (HEMIN). In contrast, gill structure (GABNO) changes were significantly lower (12%) in oysters from treatment DM (Chi-squared=7.46) compared to the others treaments. The three treatments ST, DS and DM were significantly different at α=1% when all pathological abnormalities were simultaneously considered.

Table 6. Count of the presence of gill or mantle abnormalities from the treatments: ST: starvation condition; DS: *Skeletonema costatum;* DM: mixed algal diet. Not significant: NS; significant at 5% level (*). (BASIN) basophilic cytoplasmic inclusion bodies in connective tissue of gills and mantle; (FEULG) Feulgen positive cytoplasmic inclusion bodies in connective tissue of gills and mantle; (GABNO) Drastic changes of the normal gill structure in several areas as filament fusion; (HEMIN) mass hemocytic cellular infiltration in connective tissues of several organs.

Diets	ST	DS	DM	Chi 2	Sig. level (1)
BASIN	10	6	14	5.48	NS
FEULG	10	15	18	5.64	NS
GABNO	2	10	11	7.46	*
HEMIN	21	22	21	0.23	NS
MACRO	16	14	12	4.35	NS

Multivariate Analysis

The correlation matrix showed positive relationships among the physiological variables FECES and RESPI and condition index (CONDI) and between FECES and the consumption rate (CONSU) (table. 7). In contrast, no relationship was established between CONSU and RESPI, nor between macroscopical abnormalities (MACRO) and other variables. Several variables were highly correlated: basophilic inclusion bodies (BASIN) and Feulgen positive corpuscles (FEULG),

and Feulgen positive corpuscles and gill structure abnormalities (GABNO) detected by histology Gill structure changes observed by histological examination were not correlated with basophilic inclusion bodies. Negative relationships were observed between histological abnormalities (GABNO, BASIN and FEULG) and physiological characteristics (RESPI, CONSU and FECES), but the clearance rate (CLEAR) was positively correlated with gill structure abnormalities (GABNO).

These results prompted us to select the following variables to perform the Principal Component Analysis: BASIN, FEULG, GABNO, DMW, DSW, CONSU and RESPI (figure 9). Four axes summarized 85% of the total variance (table 8). The RESPI and DMW characterized the first axis, while BASIN and FEULG the second, CONSU the third and GABNO the fourth axis (figure 9).

Table 7. Correlation matrix: (BASIN) basophilic cytoplasmic inclusion bodies in connective tissue of gills and mantle; (FEULG) Feulgen positive cytoplasmic inclusion bodies in connective tissue of gills and mantle; (GABNO) Drastic changes of the normal gill structure in several areas as filament fusion; (MACRO) gills abnormalities detected through macroscopical examination; (CONDI) condition index; (CLEAR) clearance rate; (CONSU) consumption rate; (FECES) feces production; (RESPI) respiration. *Circled values indicate significant level. * : 0.05 level; ** : 0.01 level and *** : 0.001 level.*

	gabno	macro	basin	feulg	condi	clear	consu	feces	respi
gabno		0.03	0.15	(0.31)	-0.24	(0.29)	-0.09	-0.11	(-0.27)
macro	NS		-0.12	-0.12	-0.18	0.11	0.02	-0.05	0.10
basin	NS	NS		(0.68)	-0.01	0.03	(-0.29)	-0.16	-0.07
feulg	**	NS	***		-0.13	0.06	-0.24	(-0.29)	-0.21
condi	NS	NS	NS	NS		-0.03	0.14	(0.37)	(0.67)
clear	*	NS	NS	NS	NS		(0.40)	0.22	0.00
consu	NS	NS	*	NS	NS	**		(0.66)	0.17
feces	NS	NS	NS	*	**	NS	***		(0.36)
respi	*	NS	NS	NS	***	NS	NS	**	

Table 8. Eigenvalues and percentage of variance explained by the principal axes of PCA.

Axis	Eigenvalues	%	cumulative
1	2.53	36.1	36.1
2	1.72	24.5	60.6
3	0.98	14.1	74.7
4	0.72	10.3	85.0
5	0.53	7.7	92.7
6	0.26	3.7	96.4
7	0.25	3.6	100.0

DISCUSSION

Condition index ranged from 1.5 to 5.5 during a rearing cycle for *Crassostrea gigas* in the Bay of Marennes-Oléron (Bodoy *et al.*, 1986). In this experiment, oysters from the starved treatment ST reached a 2.74 value, well above the minimum value encountered in natural conditions. The condition index for ST group was 3.15 when the experimentation started. Therefore, while seawater temperature increased from 14.5°C to 21°C, the condition index of ST oyster group was not significantly affected by starvation. However, the complete starvation did not occur since bacteria and total particulate matter (1.0-1.7 mg l^{-1}), circulating through the biological filter, represented available food for the oysters.

The 12.4% lipid content estimated in DM treatment oysters characterized a mature stage (Deslous-Paoli and Héral, 1980). Moreover, the glycogen storage percentage for group DS animals typifies the stage of the maturation process (Gabbott, 1975). With regards to protein content, all the estimates are below those from wild oyster populations in spite of similar analytical methods being used. Although the protein content usually increases between February and May, we reported an abnormal 10% loss in treatment DS. This pattern is likely to represent stress resulting from the *Chlamydia*-like infection. Differences in carbohydrate and glycogen content, depressed in the DM group, probably resulted from the gonadal maturation process.

The allometric coefficient for oyster respiration was used for our experimental populations since consumption depends mainly on the individual whole weight (Bayne et Newell, 1983) (i.e. gonadal and somatic tissues). Therefore, a weight increase due to reproductive effort is likely to result in respiration increase. In contrast, the clearance rate' allometric coefficient is inappropriate during reproductive growth, since it depends mainly on gill surface area. Therefore, the use of this allometric coefficient is unlikely suitable to estimate clearance rate during the maturation process.

Clearance and food consumption rates are therefore probably underestimated for the treatment DM and to a lesser extent for treatment DS, resulting in underestimated scope for growth. This effect of the maturation process on allometric coefficients has been pointed out by Bayne et Newell (1983), Ansell (1973) and Emerson *et al.*, (1988) respectively on *Mytilus edulis*, *Donax vittatus* and *Mya arenaria*.

Moreover, the methodology developed for the "instantaneous" scope for growth measurement may have mainly affected the sexually mature oysters (group DM) than the other treatments. By way of example, allometric coefficients to standardize the respiration results are significantly different between ripe or immature oysters. The poor fittings of the curve between the biomass production and the scope for growth was unexpected for treatment DM after one month on an improved food quality.

While several authors discuss the energy balance and allocation during the maturation process, little evidence is available on the feeding behavior during the spawning period. Newell and Thompson (1984) reported a reduced clearance rate for *Mytilus edulis* between 5 to 10 days during the spawning period. Also, *Crassostrea virginica* sperm is likely to include an hormone-analogue which expands the ostia of the gills to a size range greater than the gametes and to reduce retention rate during spawning (Nelson and Alison 1940, in Newell and Thompson, 1984).

Although not a complete starvation, the ST treatment resulted in negative scope for growth. Those oysters were significantly more affected by the *Chlamydia*-like microorganism than where otherwise treated. A Chi squared test performed on all pathological criteria (BASIN, FEULG, GABNO and HEMIN) was significant at 5 % level value. The oyster fed with algae (DS and DM) had a positive scope for growth. The DS oysters fed with *Skeletonema* sp. initiated the maturation process. The condition DM, oysters fed with mixed algae had already reached sexual maturity when physiological functions were estimated. The reduced scope for growth in treatment DM does not reflect biomass production (P), which were results significantly higher for this group compared to the others groups. This difference can be explained by biological feeding behavior associated

with gonadal maturation stage and/or, by some bias resulting from inappropriate allometric coefficient used for physiological computations.

The production (P) as defined by Winberg (1960), and the scope for growth (SFG) (Warren and Davis, 1967) are two representations of the same concept. The SFG reflects the instantaneous bivalve capacity to use quality and quantity of food, evaluated in energetic terms, under controlled conditions. Therefore, it represents the potential growth. In contrast, the biomass production integrates the whole environmental variability over the beginning and the experiments completion representing the effective growth. For monitoring networks using bioindicators, such as the Mussel Watch' in the USA, or the 'RNO' in France, both approaches have been considered. Gilfillan and Vandermeulen (1978), Bayne and Worall (1980), Thompson and Mac Donald (1991) reported no significant differences between scope for growth under laboratory experimental conditions and natural production. Moreover, Bayne *et al.*, (1985) reached similar conclusions when physiological measurements were carried out within 24 hours after bivalve sampling (Widdows, 1985).

In this study, the scope for growth was measured using a standard water with the algae *Tetraselmis suecica* (10000 cel ml^{-1}). The SFG was 10 J h^{-1} g^{-1} and 25 J h^{-1} g^{-1} for treatment DM and DS respectively. Although absorption rates were almost similar between the two groups, oysters from the DM treatment had a lower clearance and consumption rates than those from DS treatment. Actually, the total production including somatic and gonadic tissues, and shell organic matrix was estimated to 0, 10.1, and 45.8 J h^{-1} g^{-1}, respectively at the end of the experiment. These results differ considerably from SFG results: - 22.6, 25.1 and 9.8 J h^{-1} g^{-1} for the conditions ST, DS and DM respectively.

Several criteria may be considered as stress indicators. Barber *et al.*, (1988), Newell (1985) and Newell and Barber (1988) have shown that clearance rate, oxygen consumption or condition indices were appropriate bioindicators for assessing effects of the pathogen *Haplosporidium nelsoni* on the physiological condition of the Eastern oyster *C. virginica* . Our results confirm this approach assessing pathogen effects. One group of oysters, with damaged gills, was well identified when fitted to the model as showing 'abnormal' clearance rate behavior (figure 8).

Photonic and electronic histological examinations showed abnormalities linked to the occurrence of the *Chlamydia*-like organism in all three treatments (ST, DS and DM). The basophilic cytoplasmic inclusion bodies detected by using Hemalum-Eosin stains are considered to be *Chlamydia*-like organisms.

Among the four abnormalities detected by photonic histology, gill structure changes seemed to be treatment dependent. Oysters from treatment ST appeared to be more heavily infected with *Chlamydia*-like organisms than those from the others treatments. No significant difference in prevalence was observed among oysters from treatment DM and DS. Based on these results, it would be interesting to test the *Chlamydia*-like infection trend on a greater experimental time. These abnormalities in a large number of oysters among the three treatments did not facilitate discriminating these various treatments.

In our study, a significant relationship between gross and histological examinations was not established. In several cases, macroscopic lesions were not associated with histological abnormalities. This might be explained by technical constraints since only one 3 µm section was observed per oyster. On the other hand, cases in which microscopic examinations revealed tissue abnormalities without the presence of macroscopic lesions may represent an early stage of infection.

The significant relationship between the occurence of basophilic inclusion bodies and the presence of Feulgen positive corpuscles suggest that both types of abnormalities are similar and dependent upon the stain used (i.e., Hematoxylin and Eosin, Feulgen and Rossenbeck nucleal reaction). This evidence confirms results from Renault *et al.*, (1994). The basophilic inclusion bodies also positively stained with Feulgen is stain and indicate the presence of DNA within inclusions.

The positive correlation between Feulgen positive inclusion bodies (FEULG) and gill structure lesions (GABNO) detected by histology demonstrated that microorganisms might induce tissue

degradation. However, the occurrence of basophilic corpuscles (BASIN) was not correlated to gill structure changes (GABNO). Several hypotheses could be proposed:

- Nucleal reaction of Feulgen and Rosenbeck might be more sensitive than the Hematoxylin and Eosin stain for *Chlamydia*-like infection detection. Moreover, gill structure changes might be easier to detect than basophilic inclusion bodies occurrence.

- Feulgen positive corpuscles might be cellular fragments containing post-necrosis DNA and therefore, associated with drastic changes of tissue structure at histological level.

One of the main objectives of this study was to examine whether a relationship exists between physiological activities and pathological conditions. With regards to pathology results, the weakness of the starved oysters (ST) was well demonstrated. The positive correlation between the Feulgen reaction and the presence of basophilic inclusion confirmed the presence of microorganisms in gill and mantle connective tissues.

Incorporating absorption rate or scope for growth variables into the correlation matrix, did not improve the relationships between physiology (e.g. respiration, clearance) and pathogenicity. Also, no correlation was established between macroscopical observations and physiological functions.

Several negative correlations (at 5% significance level) were associated most of the pathological and physiological criteria. In contrast, the clearance rate was positively correlated to histological gills abnormalities. Comparison of measurements and clearance model estimates confirmed theses results. Therefore, *Chlamydia* like infections can be detected through clearance rate measurements on oysters affected by gill abnormalities. We suggest that in order to sustain their standard metabolism, these infected oysters compensate for the deficit in functional gill surface by increased clearance activity.

In marine molluscs, various intracellular prokaryotes have been described in many species. Several authors have linked infection to abnormal mortality: Buchanan (1978) in *Tellina tenuis*; Comps and Raimbault (1978) in *Donax trunculus*; Elston (1986) in *Siliqua patula*; Gulka *et al.*,(1983) in *Placopecten magellanicus* and Legall *et al.*, (1988) in *Pecten maximus*. However, a direct link between microorganism and abnormal mortality has not yet been identified, excepted in the case of a rickettsia infection on the common scallop, *Pecten maximus* (Le Gall *et al.*, 1991). In this case, a physiological study pointed out the energetic loss due to the rickettsia infection that affected the overall energy balance.

Our study demonstrates that simple physiological measurements, like clearance rate, can detect gill malfunction. The bioindicator role through a simple physiological function assessment is critical to detect stress or pathogens in oysters. Although no relationship was established between mortality and pathogen occurrence. Our experiment demonstrated that diet composition affected infection level, and therefore migh be critical in terms of overall shellfish management, when carrying capacity is limited by biomass overstocking (case of the cultures in Marennes-Oléron Bay). Oyster condition has been significantly affected due to overstocking since introduction of the Pacific oyster into this Bay during the 1970s (Grizel and Héral, 1991). Therefore, our study provides insight for improving shellfish management so as to maximize food availability and thus, limit pathogen effects on the oyster population. In the near future, pathogen culture should allow experimental infection to assess quantitatively the pathogen impact on oysters.

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