**Universidade de Évora**

Departamento de Biologia

Mestrado de Biologia e Ecologia do Litoral Marinho

# Effects of field metal contamination on sperm motility and RNA/DNA ratio in two echinoderm species

## Efeito da acumulação de metais pesados na motilidade do esperma e no índice ARN/ADN de populações de duas espécies de equinodermes

Ana Isabel dos Ramos Catarino

Orientação:
Dr. Philippe Dubois (Université Libre de Bruxelles, Bélgica)
Dr. Henrique Nogueira Cabral (Faculdade de Ciências da Universidade de Lisboa, Portugal)

*Esta dissertação não inclui as críticas e sugestões feitas pelo júri*

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# Contents

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## ABSTRACT

**Effects of field metal contamination on sperm motility and RNA/DNA ratio in two echinoderm species**

This study aimed to assess the effects of field metal contamination on sperm motility and RNA/DNA ratio in two populations of echinoderms: *Asterias rubens* and *Echinus acutus*. These species occur naturally along a contamination gradient of cadmium, copper, lead and zinc, in a Norwegian fjord (Sørfjord). Sperm motility was quantified with the help of a computer assisted sperm analysis system (CASA). The RNA/DNA ratios were assessed based on a 1-dye, (Ethidium bromide)/1-enzyme (RNase) 96-well microplate fluorometric assay. Although both species appeared to readily accumulate metals, neither sperm motility parameters in *A. rubens* nor RNA/DNA in the body wall of both species were affected. The RNA/DNA ratios in *A. rubens* pyloric caeca were significantly higher in the most contaminated station. Even thought this fjord is considered a highly contaminated place, these populations seem to tolerate these metal contamination levels.

## RESUMO

**Efeito da acumulação de metais pesados na motilidade do esperma e no índice ARN/ADN de populações de duas espécies de equinodermes**

Este estudo teve como objectivo verificar o efeito da acumulação de metais pesados na motilidade do esperma e na razão ARN/ADN em indivíduos de duas populações de equinodermes: *Asterias rubens* e *Echinus acutus*. Estas espécies ocorrem naturalmente ao longo de um gradiente de contaminação de cádmio, cobre, chumbo e zinco, num fiorde norueguês (Sørfjord). A motilidade do esperma foi quantificada com o auxílio de um sistema informático especializado (CASA). A razão RNA/DNA foi determinada através de ensaios utilizando brometo de etídio como corante fluorescente. Embora se tenha verificado acumulação de metais pesados nos tecidos dos indivíduos, não se registaram diferenças significativas na motilidade do esperma e na razão ARN/ADN do tegumento em função do grau de contaminação do ambiente. Apenas os valores da razão ARN/ADN do ceco pilórico de *A. rubens* foram significativamente maiores na estação mais contaminada. Estas populações parecem então tolerar os níveis de contaminantes existentes no Sørfjord.

# INTRODUCTION

Echinoderms are considered good environmental indicators, because they exhibit a high sensitivity to environmental changes, have a sedentary way of life and integrate local pollution (Guillou et al., 2000; Coteur et al., 2003a). In particular, it has been shown that they accumulate metals as a function of the contamination level of the environment (e.g. Warnau et al., 1998; Guillou et al., 2000; Coteur et al., 2003b and references therein). Some are also considered to be key-species, playing a structuring role in benthic ecosystems (Menge et al., 1994). Therefore, any effect of a contaminant on populations of these species may affect the whole community. In the last decades the use of echinoderm, like sea urchins and starfish, has been strongly recommended by ecotoxicologists (Dinnel et al., 1987, 1988; 1989; Coteur et al., 2003a) and international environmental agencies (e.g. US EPA, 1994; Environment Canada, 1997).

The Sørfjord, located in Southwest Norway, holds at its head three large metal smelters. For more than 80 years their waste was discharged into this fjord, contaminating severely its sediments and making the Sørfjord one of the most polluted fjords in the world. The discharges only started to be reduced from 1986 on and in 1992 remedial actions also took place: the metal containing sludge started to be deposited into mountain caves and sediments in shallow water, close to one of the smelters, were capped by an impermeable membrane in order to limit metal release (see Coteur et al., 2003b; Gillan et al., 2005; Ruus et al., 2006 and references therein). Although remedial actions have been taken in recent years and discharges into the fjord were extremely reduced, the Sørfjord still remains one of the most contaminated areas in Europe (OSPAR Commission, 2000). A steep metal contamination gradient occurs from the head towards the opening into the Hardangerfjord (Coteur et al. 2003b).

In this study two echinoderm species, *Asterias rubens* and *Echinus acutus*, that occur all along the Sørfjord, were chosen to assess heavy metal contamination effects in two well known biomarkers: sperm motility and RNA/DNA ratio.

Experimental gamete exposure to metals is known to affect echinoderms sperm quality, the ability of sperm to successfully fertilize an egg, and embryogenesis (Warnau et al., 1996; His et al., 1999; Larrain et al., 1999). Given that sperm motility (progressive movement) is a requirement for fertilization and is correlated with

fertilization success, it is generally used to assess sperm quality (Kime et al., 2001; Rurangwa et al., 2004). Sperm motility can me measured either by an observer or automatically. The first methodological approach usually has a higher degree of subjectivity associated, mainly due to the choice of the observers, their experience and their interpretation of movement. Computer assisted sperm analysis systems, known as CASA, provide much more accurate and reliable results (Rurangwa et al., 2004). A CASA system refers to the physical equipment used to visualize and digitize static and dynamic sperm images, as well as to the methods used to process and analyse them. A simple sperm movement video analysis provides standardize results with high reproducibility (WHO, 1999; Kime et al., 2001; Rurangwa et al., 2004). The CASA system has been extensively used in fish (Kime et al., 2001; Rurangwa et al., 2004), but also in echinoderm sperm analysis (Au et al., 2000; Au et al., 2001; Lu and Wu, 2005).

In laboratorial studies it has been demonstrated, with the help of the CASA, that echinoid sperm motility decreased with metal exposure, either from sperm itself (Au et al., 2000) or adults chronic contamination (Au et al., 2001). These results suggest that sperm motility studies may be a good approach in evaluating echinoderm reproductive impairment by field metal contamination.

The RNA/DNA ratio has been used as a biochemical growth rate indicator, providing growth rates and metabolic status estimates of a wide range of marine organisms, for instance larval fishes (Buckley et al., 1999), crustaceans (Rosa and Nunes, 2003), molluscs (Melzner et al., 2005), corals (Meesters et al., 2002) and echinoderms (Watts and Lawrence, 1990; Frantzis et al., 1992; Liyana-Pathirana et al., 2002). This index has been proven to be sensitive to environmental changes (Meesters et al., 2002; Rosa and Nunes, 2003) and positively correlated with protein synthesis and growth (Barron and Adelman, 1984; Wang and Stickle, 1988; Rosa and Nunes, 2003). Higher growth rates are expected to enhance total ribonucleic acid (RNA) concentrations, hence the ratio of RNA to deoxyribonucleic acid (DNA). The RNAs are necessary for the biosynthesis of proteins and their number and activity varies in response to the requisite for protein synthesis. The DNA levels in cells remain rather constant and, thus, are used to normalize the measured RNA concentrations. Hence, the RNA/DNA ratio is an index of cell's metabolic rate (Buckley et al., 1999).

Since the 1970s this ratio has been broadly used, although different methods have been developed. The first method to be used was the UV-based nucleic acid analysis. However, now-a-days mainly spectrofluorometric nucleic acid analysis is used, which is

considered a more sensitive and rapid method and as precise as the former one (Buckley et al., 1999; Caldarone et al., 2001). The most commonly spectrofluorometric method used employs the fluorophore ethidium bromide (EB: 3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide), which binds to nucleic acids, giving a measure of the total sample amount of them. The enzyme RNase is then added, in order to digest RNA, being the remaining fluorescence attributed to DNA and thus providing also a way to back calculate the RNA amount (Buckley et al, 1999; Caldarone et al., 2001).

Growth impairment due to contaminant exposure has been evidenced by a decline of this ratio in fish, crustaceans and molluscs (Barron and Adelman, 1984; Wang and Stickle, 1988; Miliou et al., 1998; Wo et al., 1999; Adham, 2002). These data strongly support the use of the RNA/DNA ratio to assess the effects of contamination on growth rate in echinoderms.

In the recent years, an increasing emphasis has been dedicated to the evaluation of contamination effects in marine organisms. Several European and worldwide legislative documents have introduced monitoring and environmental assessment studies as a way of diagnosis and remedial measures development. The present study aimed to evaluate the effects of heavy metal contamination in two species of echinoderms in a heavily contaminated fjord in Norway, and thus to contribute to a better understanding of this issue.

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# Effects of field metal contamination on sperm motility and RNA/DNA ratio in two echinoderm species

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**Abstract**

This study aimed to assess the effects of field metal contamination on sperm motility and RNA/DNA ratio in echinoderms. In order to do so, populations of *Asterias rubens* and *Echinus acutus*, that occur naturally along a contamination gradient of cadmium, copper, lead and zinc, in a Norwegian fjord (Sørfjord), were studied. Sperm motility, a measure of sperm quality, was quantified using a computer assisted sperm analysis system (CASA). The following motion parameters of individual sperm cells were assessed: curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP). The RNA/DNA ratios were assessed based on a 1-dye, (Ethidium bromide, EB)/1-enzyme (RNase) 96-well microplate fluorometric assay. Although both species appeared to readily accumulate metals, neither sperm motility parameters in *A. rubens* nor RNA/DNA ratio in the body wall of both species were affected. The RNA/DNA ratios in *A. rubens* pyloric caeca was significantly higher in the most contaminated station. However, this was highly dependent on a few possible outliers and when these values were removed from analysis, significant differences disappeared. Even thought the Sørfjord is considered a highly contaminated place, populations of *A. rubens* and *E. acutus* seem to be able to survive under these conditions and possibly to reproduce.

**Keywords:** *Asterias rubens*; *Echinus acutus*; Sørfjord; metal contamination; sperm motility; RNA/DNA ratio

## 1. Introduction

Numerous echinoderms play a structuring role in benthic ecosystems where they act as key-species (Menge et al., 1994). For that reason, any effect of a contaminant on populations of these species may affect the whole community. It is well known that echinoderms readily accumulate heavy metals and effects from molecular to individual levels have been documented both in laboratory and field conditions (Temara et al., 1997c; Warnau et al., 1998; den Besten et al., 2001; Coteur et al., 2003a,c; Danis et al., 2004). Experimental exposures provide readily interpretable results and trustworthy cause-effect relationships between metal contamination and impacts on echinoderms, namely on reproduction, steroid metabolism, skeletal growth and immune function (Voogt et al., 1987; den Besten et al., 1989, 1991; Temara et al., 1997b; Au et al., 2001; Coteur et al., 2001, 2005). Effects of metals in field contaminated populations are diverse. On the one hand, alkaline phosphatase activity in digestive organs may be significantly reduced (Temara et al., 1997a,c), the immune system can be either depressed or stimulated (Coteur et al., 2003a,b) and impaired development of the offspring have been related to metal contamination of the progenitors (Guillou et al., 2000). On the other hand, populations of starfish can maintain stable populations in heavily contaminated environment with no apparent effects on eggs and fertilization rates (Temara et al., 1998; Dubois et al., 2004).

In the present study the effects of metals in field contaminated populations of the starfish *Asterias rubens* were assessed using two sensitive physiological aspects that are crucial for the maintenance of populations: sperm motility and protein synthesis, thus growth. The latter was also investigated in the sympatric sea urchin *Echinus acutus*.

Sperm motility (progressive movement) is a requirement for fertilization and is correlated with fertilization success. It is generally used to assess sperm quality (Au et al., 2001; Rurangwa et al., 2004). Furthermore, *in vitro* exposure of gametes to metals is known to affect sperm quality (Au et al., 2001) and embryogenesis (Warnau et al., 1996) of echinoderms, a fact that has led several environmental agencies to use these assays as standard procedures (e.g. US EPA, 1994; Environment Canada, 1997). However, the effects of field metal contamination on echinoderm sperm quality have never been investigated.

The RNA/DNA ratio has been used as a biochemical growth rate indicator, providing growth rates and metabolic status estimates of a wide range of marine

organisms, for instance fish (Barron and Adelman, 1984; Adham, 2002; Fonseca et al., 2006), crustaceans (Wang and Stickle, 1988), molluscs (Melzner et al., 2005), cnidarians (Meesters et al., 2002) and echinoderms (Watts and Lawrence, 1990; Frantzis et al., 1992; Liyana-Pathirana et al., 2002). The use of this ratio is based on the assumption that the amount of DNA, in a given organism, is relatively constant under changing environmental conditions, since it is mainly a function of cell number, whilst total RNA, which is a function of ribosome number, is positively correlated with protein synthesis, and thus, growth. Metal exposures resulted, in numerous invertebrates and fish, in a decrease of RNA/DNA ratio (Barron and Adelman, 1984; Wang and Stickle, 1988; Adham, 2002).

The present work was carried out in the Sørfjord, a southern branch of the Hardangerfjord, located in Western Norway. Three smelters, built at the head of the fjord, have discharged their wastes into it for more than 80 years, generating a hot spot of metal contamination (see Temara et al. 1998 and references there in). Inputs were significantly reduced in 1986 and followed by remediation actions (see Coteur et al., 2003c; Gillan et al., 2005 an references there in). However, metals are trapped in sediments of the fjord, limiting net efflux to the open sea, but allowing some internal recycling. The release of these sediment-associated metals is significant and is stimulated by the activity of the benthos (see Temara et al., 1998 and references there in). Populations of *A. rubens* and *E. acutus*, occur naturally all along the fjord, including in the most contaminated sites. In these sites, *A. rubens* is effectively contaminated by metals (Coteur et al 2003c). Metal contamination of *E. acutus* in the Sørfjord has never been assessed.

## 2. Materials and Methods

### 2.1 Study Area and Sampling

Sampling took place in the Sørfjord (Fig. 1), which is 37 km long, 1-2km wide and 390m maximum depth. Individuals were collected during March 2006, in shallow water, by SCUBA diving in 5 different stations at increasing distances from the fjord head: S1, S2, S3 and S4 for *Asterias rubens* and S1, S2, S3 II and S4 for *Echinus acutus* (Fig. 1; Table 1). The S4 station was located just outside the Sørfjord, in the Hardangerfjord, towards the open sea. In order to avoid exposure to the vertical gradient of salinity and, therefore, to an osmotic shock, the animals were placed into sealed jars filled with their surrounding water before being brought to the surface. These were aerated while transported to the laboratory located in Aga (Fig. 1). Samples of the top 5cm layer of sediments were collected in polyethylene containers, in all stations. These samples were stored at -20°C and then dried (60°C for 2 days) before metal analysis.

For sperm motility analysis, 20 *A. rubens* individuals were collected from each station and brought to Belgium, in aerated water, no later than 3 days after their collection. They were then installed in a closed circuit aquarium system and within two weeks sperm motility video recordings were carried out.

For RNA/DNA analysis around 25 individuals of *A. rubens* and *E. acutus* were collected per station. All individuals were processed within around 5 hours after collection. Prior to dissection, arm length of the starfish (R), from arm tip to mouth, and ambital diameter of sea urchins (D) were measured; all individuals were weighted (W). Samples of body wall and pyloric caeca, from *A. rubens*, and of body wall and gonads, from *E. acutus*, were dissected and immediately frozen in liquid nitrogen and subsequently stored at -80°C until RNA/DNA determinations. The remaining body wall, pyloric caeca and gonads from *A. rubens* and body wall, gut (after removal of its content) and gonads, from *E. acutus* were taken from the same individuals as for RNA/DNA analysis, dried at 60°C for 2 days and stored for metal analysis.

Males whose gonads were infected by ciliates (2 starfish in S1 for RNA/DNA), hermaphrodite individuals (2 *A. rubens* in S1 for RNA/DNA analysis) and individuals whose gender cannot be determined (2 *E. acutus* from S1) were not further used for both metal and RNA/DNA analysis.

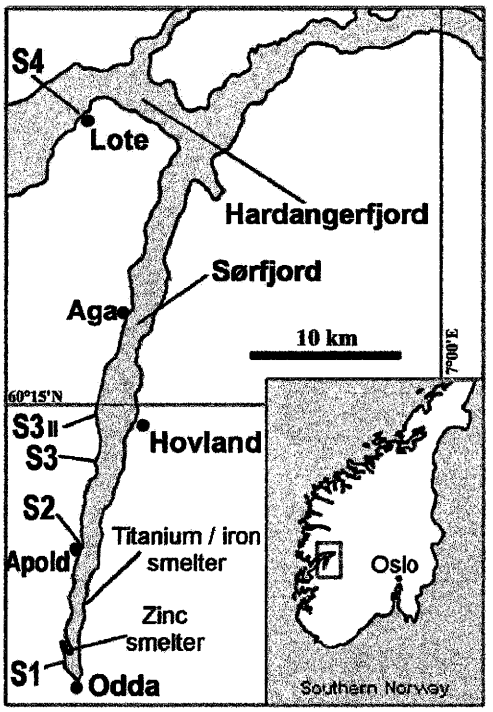


Fig. 1. Location of the sampling sites in the Sørfjord (Southwestern Norway): S1, S2, S3, S3 II, S4.

Table 1. Location and conditions of the different sampling sites in the Sørfjord, Norway. In stations S1, S2 and S4 both species were collected; on S3 only *A. rubens* was sampled whereas on S3 II it was only *E. acutus*.

| Site | Coordinates | | Distance from the head of the fjord (km) | Collection Depths (m) | Water Temperature (°C) | Salinity (‰) |
|---|---|---|---|---|---|---|
| S1 | 60°04.79 N | 06°31.81 E | 1.6 | 1.5-9.0 | 5-9 | 33-38 |
| S2 | 60°09.02 N | 06°32.76 E | 9.0 | 3.0-6.0 | 5-8 | 34-36 |
| S3 | 60°12.58 N | 06°33.80 E | 15.5 | 2.5-5.0 | 7 | 34-35 |
| S3 II | 60°14.61 N | 06°33.80 E | 19.5 | 8.0 | 6 | 35 |
| S4 | 60°24.93 N | 06°31.55 E | 39.6 | 2.0-6.0 | 4-6 | 35-36 |

## 2.2 Metal Analysis

Mineralization of samples and metal analysis were carried out as described in Coteur et al. (2003c). Cadmium (Cd), copper (Cu) and lead (Pb) were analyzed by graphite-furnace atomic absorption spectrometry (GF-AAS) and zinc (Zn) by flame atomic spectrometry (F-AAS).

## 2.3 Sperm Motility

*A. rubens* male individuals were identified by collecting some gametes through the body wall with the help of a syringe and then placed in individual aquariums containing a small amount of filtered sea water (1cm layer). Spawning was induced by injecting in the general cavity of each arm 0.2-0.4ml of 1µM 1-methyladenine solution. Spawning took place from 1h after induction and sperm was collected using a pipette, directly from the surface of the starfish, to prevent dilution with water and consequent activation, and put into a vial, placed on ice. Males whose gonads were infected by ciliates were not considered for analysis (total of 12 individuals from all stations). In order to record spermatozoa motion, 5µl of dry sperm were diluted in 8ml of filtered (0.22µm) sea water and gently mixed. Sub-samples of 150µl were immediately mounted on a glass slide chamber. Sperm movement was recorded, at room temperature, with a CCD camera (Fire-i™ 400™ Sony©) attached to a microscope, in dark-field mode. Video image scale was calibrated using a micrometer slide.

Furthermore, a sample (50µl) of diluted sperm was placed in 2450µl of 5% formol solution and then number of spermatozoa in each sample was assessed with the help of a haemocytometer.

The motility of sperm cells, previously recorded in movies, was measured using a computer assisted sperm analysis system (CASA - IVOS Sperm Analysis System, version 12, Hamilton Thorne Biosystems, Beverly MA, USA). The following motion parameters of individual sperm cells were assessed: 1) curvilinear velocity (VCL), the time average velocity of the sperm head along its actual trajectory; 2) straight line velocity (VSL), the time average velocity of the sperm head along the straight line between its first detected position and its last position; 3) average path velocity (VAP), the time average velocity of a sperm head along its spatial average trajectory. The

trajectories were computed by smoothing the actual path according to specific system algorithms. Sperm cells were considered immotile if VAP was less than $5\mu m.s^{-1}$.

The number of males used per station was: 4 for S1, 4 for S2, 3 for S3 and 1 for S4 (low numbers are due to a generally low maturity level in the populations and a high ratio of females). Video recordings used in the analysis varied between 5 and 12 per male, being usually 8, and making a total of 96: 29 video recordings for S1, 36 for S2, 23 for S3 and 8 for S4.

### 2.3 RNA/DNA ratio

Nucleic acid concentrations were determined using a 1-dye (Ethidium bromide, EB)/1-enzyme (RNase) 96-well microplate fluorometric assay, based on Caldarone et al. (2001) and on Belchier et al. (2004) protocols.

Prior to analysis, deep-frozen samples (-80°C) were freeze-dried for 48h. Around 2-3mg dry weight (DW) of pyloric caeca and gonad tissues (from *A. rubens* and *E. acutus*, respectively) were analysed, as well as around 20mg DW of body wall (from both species). Samples were extracted using Tris-EDTA buffered (5mM Tris-HCl, 0.5mM EDTA, pH 7.5) 0.01% Sodium dodecyl sulphate (SDS from SIGMA L4390). They were homogenized on ice and then centrifuged (3500g, 15 min, 4°C). From each sample, 4 aliquots of supernatant were transferred to a multi-well plate: 2 replicates for total nucleic acid determination and 2 that were enzymatically digested using RNase (Sigma R-6513; 30min at 37°C and 30min to cool down to room temperature), for DNA determination. EB was added to each well and standard curves were established for each plate using known amounts of 18S- and 28S-rRNA from calf liver (Sigma R-0889) and ultra-pure highly-polymerized calf thymus DNA (Sigma D-4764). Excitation and emission wavelengths were 365nm and 590nm, respectively. RNA fluorescence was calculated by subtracting DNA fluorescence reading to the total nucleic acid value. Sample nucleic acids concentrations were estimated by comparing fluorescence readings to those obtained from standard curves. Residual fluorescence (evaluated prior to the study by using DNase; Sigma D-4263) was considered negligible. RNA/DNA ratios were determined for each sample and expressed as $\mu g\ RNA.mg^{-1}$ sample DW divided by $\mu g\ DNA.mg^{-1}$ sample DW.

## 2.4 Statistical Analysis

Comparisons of sediments metal concentrations between stations were done using one-way ANOVA. Metal concentrations in body compartments were analysed using a two-way ANOVA (fixed factors station and gender). ANOVA were followed by multiple comparisons Tukey tests, whenever the null hypotheses were rejected (Zar, 1996).

In order to summarize the data from body compartments metal concentrations and to assess the clustering pattern of samples, a non-metric multidimensional scaling (MDS) ordination analysis was done using PRIMER-E software (Clarke and Warwick, 2001). A separate analysis was done for each species. In the case of *E. acutus*, gonads concentrations were not used since in S1 very few individuals were mature and only 3 had gonads (Table 3). Data were log(x+1) transformed and the Bray-Curtis similarity coefficient was used.

Velocity parameters (VCL, VSL and VAP) of sperm motility were analysed using mixed effects nested ANOVA (Zar, 1996). The factors used were station (fixed) and male (random, nested in the factor station). The differences sperm concentrations between stations were analysed using one-way ANOVA.

The RNA/DNA ratios from body wall and pyloric caeca from *A. rubens* were compared using 2-way ANOVA (fixed factors station and gender), followed by multiple comparisons Tukey tests (Zar, 1996). Relationships between RNA/DNA ratio in pyloric caeca and metal concentrations in the same organ were further analysed by multiple regression. The ratios from body wall and from *E. acutus* were analysed by two-way ANCOVA (fixed factors station and gender, weight as covariance). The *E. acutus* gonads were not analysed due to lack of replicates for some levels of both factors (Table 4).

Prior to all ANOVA analysis data was checked for normality and homogeneity of variances and was log(x+1) transformed whenever necessary. Except for the MDS, all statistical analyses were done using STATISTICA© software (Stat Soft, Inc, 2004) and the significance level was set at 0.05.

## 3. Results

### 3.1 Metals contamination

Metal concentrations in total sediments were analysed in the 5 stations, in order to verify the existence of a metal contamination gradient along the Sørfjord. S1 appears as the most contaminated station, contrasting with S4, where the lowest metal concentrations were observed, with the exception of Cu in S3 (Fig. 2).

The dimensions, arm length (R), of the *Asterias rubens* individuals analysed were: R = 54-100 mm; and their weight varied between 20-132g. *Echinus acutus* dimensions, ambital diameter (D), were: D = 42-100mm; weight of sea urchins varied between 40-450g. The concentrations of cadmium, copper, lead and zinc in the different body compartments are presented in Table 2 for *A. rubens* and Table 3 for *E. acutus*.

All metal concentrations in the body wall, pyloric caeca and gonads of *A. rubens* differed significantly between stations ($p<0.00001$). However, gender differences were only verified in gonads ($p<0.001$). For Cd and Pb, starfish always appeared more contaminated in S1 and less in S4. Starfish from S2 and S3 presented intermediate values except for Cd in pyloric caeca of S3 starfish which were as contaminated as those from S1. For Cu, starfish of S1 were the most contaminated while the less ones were observed in S3. Finally, Zn concentrations in *A. rubens* did not differ very much between stations (Table 2). Female gonads were generally more contaminated than males from the same station, except in the copper case.

Metal concentrations in *E. acutus* body compartments (Table 3) significantly differed between stations ($p<0.01$), except for zinc concentrations in both gut and gonads ($p>0.05$). A clear contamination gradient was only observed for Pb, with S1 presenting the highest metal contamination levels and S4 the lowest ones. For the other metals this gradient was not always so straightforward and these relations also changed according to the body compartment in question (Table 3).

In order to summarize the data provided by the heavy metal contamination of the different body compartments, two MDS analysis were preformed (one for each species). Fig. 3 shows the two-dimensional plots resultant from these analyses. In what concerns the samples from *A. rubens*, it can be seen that they tend to be grouped according to stations (Fig. 3a) and genders appear to be separated (Fig. 3b). The E. acutus samples

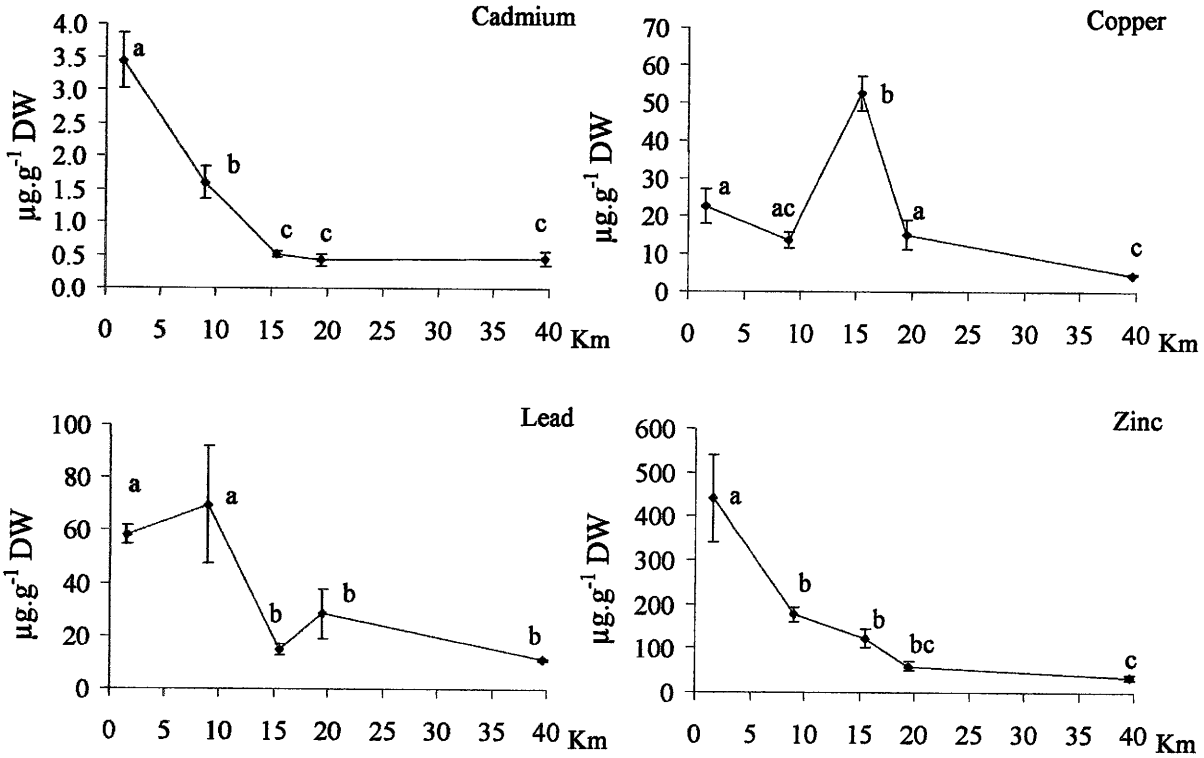


Fig. 2. Concentrations (mean ± standard deviation, n=3) of heavy metal in sediments collected at each sampling station located along the Sørfjord (see Table 1 for relation with distances from the head of the fjord). The stations that share the same letter (a-c) do not present a significant difference between their contamination levels (Tukey tests, $p>0.05$).

Table 2. Cadmium, copper, lead and zinc concentrations ($\mu g.g^{-1}$ DW; means ± standard deviation) in *A. rubens* body wall, pyloric caeca and gonads (F-females, M-males; values in brackets refer to the number of analysed individuals; values from the same body compartment with the same superscript letter (a-d) do not differ significantly from one another, based on Tukey tests, $p>0.05$).

| Heavy Metal | Body compartment | | S1 | S2 | S3 | S4 |
|---|---|---|---|---|---|---|
| Cadmium | Body wall | | 5.74 ± 2.47 (26) $^{a}$ | 3.31 ± 0.91 (30) $^{b}$ | 3.20 ± 0.86 (30) $^{b}$ | 2.81 ± 1.11 (30) $^{b}$ |
| | Pyloric caeca | | 5.49 ± 1.59 (26) $^{a}$ | 4.44 ± 1.22 (30) $^{b}$ | 5.50 ± 1.60 (30) $^{a}$ | 2.93 ± 1.04 (30) $^{b}$ |
| | Gonads | F | 0.48 ± 0.29 (19) $^{ab}$ | 0.37 ± 0.12 (17) $^{abc}$ | 0.32 ± 0.13 (15) $^{abcd}$ | 0.32 ± 0.09 (20) $^{bcd}$ |
| | | M | 0.51 ± 0.17 (7) $^{a}$ | 0.25 ± 0.08 (13) $^{cd}$ | 0.16 ± 0.06 (15) $^{d}$ | 0.18 ± 0.07 (10) $^{d}$ |
| Copper | Body wall | | 2.62 ± 0.47 (26) $^{a}$ | 2.22 ± 0.54 (30) $^{b}$ | 1.53 ± 0.40 (30) $^{c}$ | 2.38 ± 0.59 (30) $^{ab}$ |
| | Pyloric caeca | | 58.56 ± 34.09 (26) $^{a}$ | 26.70 ± 10.98 (30) $^{b}$ | 19.25 ± 8.05 (30) $^{c}$ | 26.75 ± 10.33 (30) $^{b}$ |
| | Gonads | F | 7.57 ± 2.79 (19) $^{a}$ | 4.35 ± 0.84 (17) $^{c}$ | 3.94 ± 0.65 (15) $^{c}$ | 4.36 ± 0.55 (20) $^{c}$ |
| | | M | 8.73 ± 4.30 (7) $^{a}$ | 5.96 ± 1.42 (13) $^{ab}$ | 4.68 ± 0.58 (15) $^{bc}$ | 5.09 ± 0.39 (10) $^{bc}$ |
| Lead | Body wall | | 36.84 ± 10.15 (26) $^{a}$ | 22.07 ± 4.54 (30) $^{b}$ | 8.74 ± 1.15 (30) $^{c}$ | 2.85 ± 0.53 (30) $^{d}$ |
| | Pyloric caeca | | 10.39 ± 3.05 (26) $^{a}$ | 6.68 ± 2.18 (30) $^{b}$ | 5.29 ± 1.18 (30) $^{c}$ | 1.71 ± 0.38 (30) $^{d}$ |
| | Gonads | F | 13.48 ± 7.95 (19) $^{a}$ | 9.79 ± 3.49 (17) $^{a}$ | 3.05 ± 1.41 (15) $^{bc}$ | 2.37 ± 0.75 (20) $^{c}$ |
| | | M | 8.23 ± 6.02 (7) $^{ab}$ | 4.14 ± 2.01 (13) $^{bc}$ | 1.61 ± 1.61 (15) $^{c}$ | 0.91 ± 0.30 (10) $^{c}$ |
| Zinc | Body wall | | 387.88 ± 133.25 (26) $^{a}$ | 284.31 ± 94.23 (30) $^{b}$ | 278.25 ± 132.99 (30) $^{b}$ | 229.15 ± 87.07 (30) $^{b}$ |
| | Pyloric caeca | | 81.54 ± 25.67 (26) $^{c}$ | 124.16 ± 25.26 (30) $^{a}$ | 116.11 ± 21.62 (30) $^{ab}$ | 100.30 ± 23.83 (30) $^{b}$ |
| | Gonads | F | 524.67 ± 218.38 (19) $^{a}$ | 309.53 ± 81.74 (17) $^{ab}$ | 235.44 ± 89.11 (15) $^{b}$ | 321.94 ± 70.61 (20) $^{ab}$ |
| | | M | 222.08 ± 175.53 (7) $^{bc}$ | 127.16 ± 86.89 (13) $^{cd}$ | 106.99 ± 97.44 (15) $^{d}$ | 117.21 ± 87.64 (10) $^{cd}$ |

Table 3. Cadmium, copper, lead and zinc concentrations ($\mu g.g^{-1}$ DW; means ± standard deviation) in *E. acutus* body wall, gut and gonads (values in brackets refer to the number of analysed individuals; values from the same body compartment with the same superscript letter (a-d) do not differ significantly from one another, Tukey tests, $p>0.05$).

| Heavy Metal | Body Compartment | S1 | S2 | S3 II | S4 |
|---|---|---|---|---|---|
| Cadmium | Body wall | 2.77 ± 0.59 (18) $^{a}$ | 1.28 ± 0.28 (20) $^{b}$ | 1.36 ± 0.26 (20) $^{b}$ | 0.84 ± 0.17 (20) $^{c}$ |
| | Gut | 4.50 ± 6.30 (9) $^{b}$ | 10.86 ± 2.51 (10) $^{a}$ | 7.33 ± 2.66 (10) $^{b}$ | 8.75 ± 2.16 (10) $^{b}$ |
| | Gonads | 1.15 ± 0.35 (3) $^{a}$ | 0.43 ± 0.24 (20) $^{b}$ | 0.67 ± 0.37 (18) $^{ab}$ | 0.55 ± 0.36 (20) $^{b}$ |
| Copper | Body wall | 2.42 ± 0.66 (18) $^{a}$ | 0.72 ± 0.26 (20) $^{b}$ | 0.90 ± 0.41 (20) $^{b}$ | 0.88 ± 0.25 (20) $^{b}$ |
| | Gut | 28.82 ± 21.76 (9) $^{ab}$ | 47.93 ± 24.09 (10) $^{a}$ | 14.45 ± 3.92 (10) $^{b}$ | 29.01 ± 12.11 (10) $^{ab}$ |
| | Gonads | 2.66 ± 1.84 (3) $^{ab}$ | 3.27 ± 0.87 (20) $^{a}$ | 4.50 ± 1.86 (18) $^{b}$ | 3.48 ± 1.10 (20) $^{ab}$ |
| Lead | Body wall | 13.00 ± 5.85 (18) $^{a}$ | 4.86 ± 2.97 (20) $^{c}$ | 6.36 ± 1.16 (20) $^{b}$ | 3.03 ± 0.63 (20) $^{d}$ |
| | Gut | 15.09 ± 5.23 (9) $^{a}$ | 12.19 ± 3.14 (10) $^{a}$ | 6.40 ± 1.08 (10) $^{b}$ | 1.89 ± 0.60 (10) $^{c}$ |
| | Gonads | 6.05 ± 0.45 (3) $^{a}$ | 0.92 ± 0.48 (20) $^{b}$ | 0.95 ± 0.21 (18) $^{b}$ | 0.84 ± 0.21 (20) $^{b}$ |
| Zinc | Body wall | 17.02 ± 5.56 (18) $^{a}$ | 8.56 ± 6.49 (19) $^{ab}$ | 7.73 ± 2.87 (20) $^{b}$ | 13.25 ± 4.45 (20) $^{a}$ |
| | Gut | 182.52 ± 48.69 (9) | 166.94 ± 44.21 (10) | 140.85 ± 22.84 (10) | 146.42 ± 32.84 (10) |
| | Gonads | 173.58 ± 209.69 (3) | 133.79 ± 97.20 (20) | 160.42 ± 141.40 (18) | 159.31 ± 132.10 (20) |

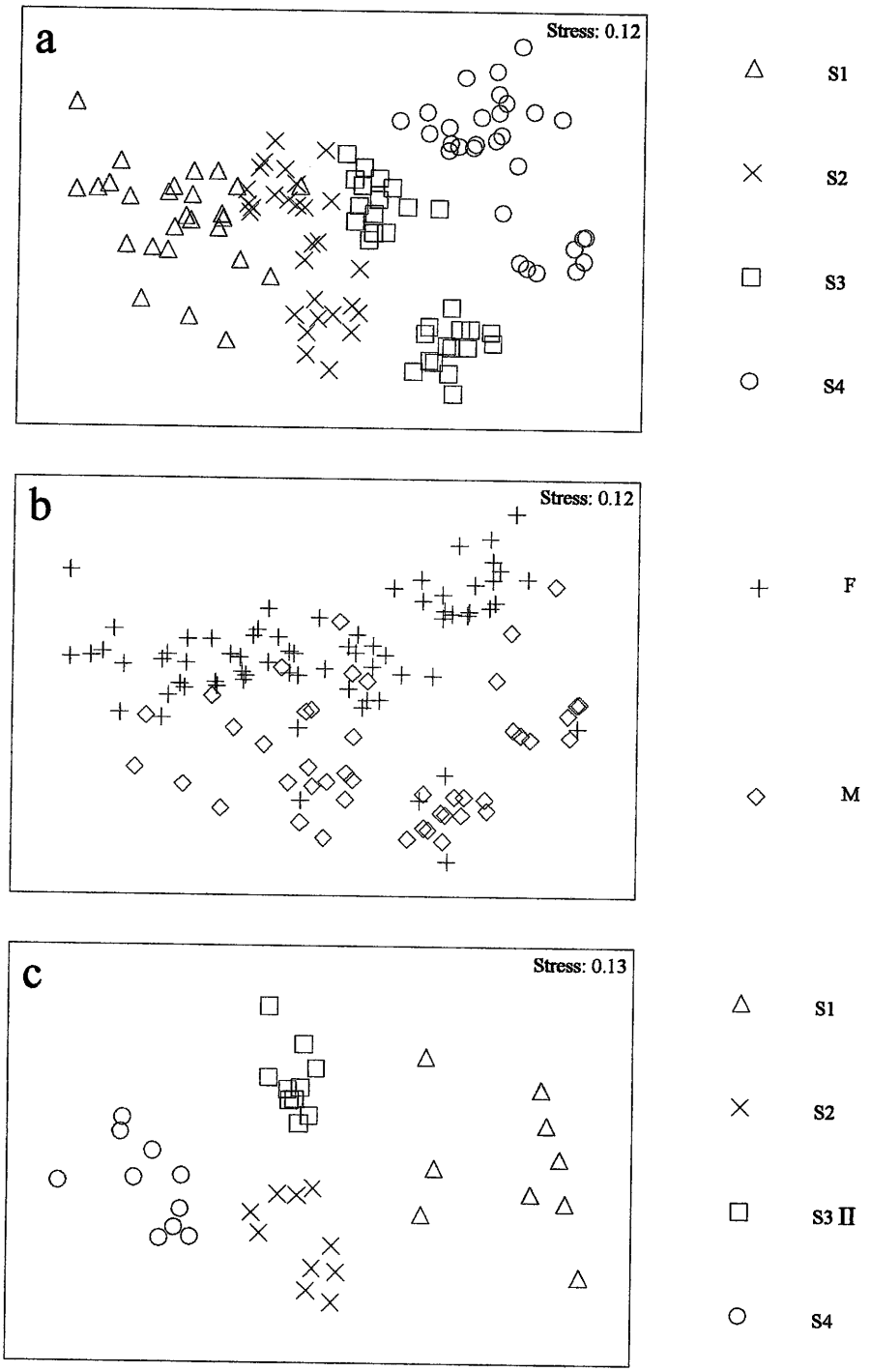


Fig. 3. Two-dimensional MDS plots of metal concentrations from the different body parts. The a) and b) plots both refer to *A. rubens* samples and show the same MDS analysis, but labelled according to the factors station and gender, respectively. The c) plot refers to *E. acutus* MDS analysis.

were also grouped according to the station in question (Fig. 3c). The stress indexes obtained for both analyses were of 0.12 and 0.13 for *A. rubens* and *E. acutus*, respectively, which indicates that the analysis gives potentially useful information about the data.

### 3.2 Sperm motility

*A. rubens* dry sperm concentrations varied between 1.04-9.75x$10^{11}$ sperm cells $ml^{-1}$. No differences between station were found ($p>0.5$). The velocity parameters (Fig. 4) varied between 193.9 and 31.4 $\mu m.s^{-1}$, for VCL, 97.5 and 26.4 $\mu m.s^{-1}$, for VSL, and 182.1 and 27.5 $\mu m.s^{-1}$, for VAP. No significant differences were found between stations ($p>0.1$).

### 3.3 RNA/DNA ratio

Table 4 shows the values obtained for both RNA and DNA concentrations, and also for RNA/DNA ratios from *A. rubens* body wall and pyloric caeca and *E. acutus* body wall and gonads.

The RNA/DNA ratios measured in *A. rubens* body wall showed no significant differences between gender and the four stations ($p>0.1$). The RNA/DNA ratios in *A. rubens* pyloric caeca significantly differed according to the stations ($p<0.005$). Multiple comparison tests showed that the effect was due to higher values in S1 males (Fig. 5). However, this was highly dependent on a few possible outliers. When these 4 values (on a total of 101) were removed, there were no significant differences ($p>0.05$). The RNA/DNA values obtained from a two-way ANCOVA for *E. acutus* body wall, showed no significantly differences between genders and the four stations ($p>0.5$)

Relationships between RNA/DNA ratio in pyloric caeca and metal concentrations in the same organ were further analysed by multiple regression. No significant relationship between Cd, Pb, and Cu concentrations and RNA/DNA ratios occurred ($p>0.5$). However, a significant negative relationship between Zn concentration and RNA/DNA ratio was evidenced ($p<0.01$)

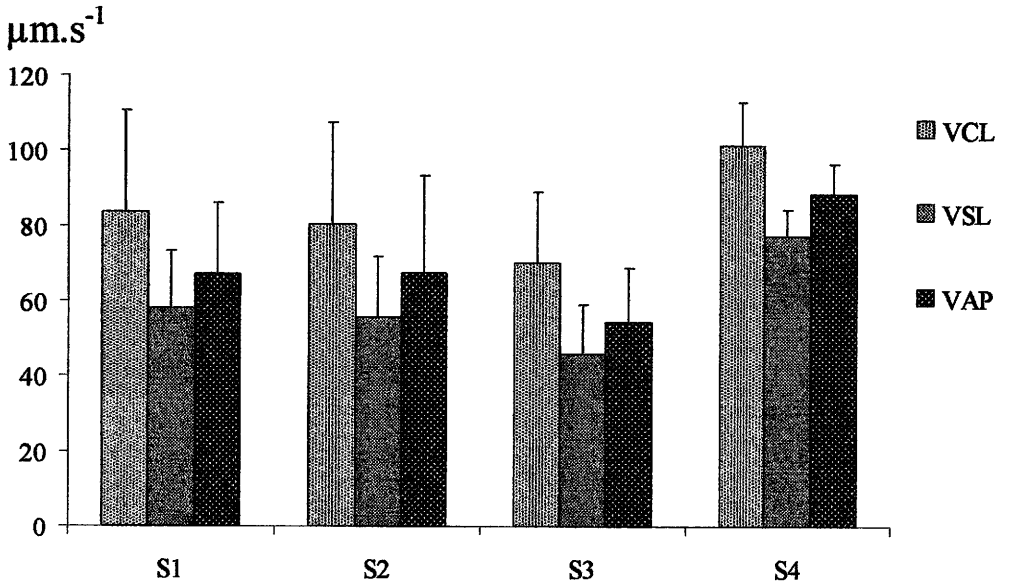


Fig. 4. *A. rubens* sperm motion parameters (mean ± standard deviation) determined in individuals collected in the four sampling stations (number of replicates: 29 for S1, 36 for S2, 23 for S3 and 8 for S4).

Table 4. RNA (μg RNA. $mg^{-1}$ DW), DNA (μg DNA. $mg^{-1}$ DW) concentrations and RNA/DNA ratios (means ± standard deviation) in *A. rubens* (body wall and pyloric caeca) and *E. acutus* (body wall and gonads) (F-females, M-males; n refers to the number of analysed individuals).

*A. rubens*

| Station | Gender | Body Wall | | | | Pyloric Caeca | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | n | [RNA] | [DNA] | [RNA]/[DNA] | n | [RNA] | [DNA] | [RNA]/[DNA] |
| S1 | F | 17 | 0.21 ± 0.09 | 0.17 ± 0.06 | 1.34 ± 0.66 | 14 | 8.00 ± 3.03 | 1.34 ± 0.66 | 6.75 ± 2.47 |
| | M | 7 | 0.28 ± 0.17 | 0.16 ± 0.04 | 1.74 ± 0.79 | 5 | 7.87 ± 2.81 | 0.94 ± 0.69 | 11.52 ± 6.11 |
| S2 | F | 17 | 0.33 ± 0.14 | 0.21 ± 0.11 | 2.02 ± 1.35 | 17 | 8.46 ± 3.66 | 1.51 ± 0.56 | 5.94 ± 2.15 |
| | M | 13 | 0.37 ± 0.20 | 0.18 ± 0.07 | 2.27 ± 1.34 | 11 | 7.32 ± 2.11 | 1.30 ± 0.60 | 6.30 ± 2.27 |
| S3 | F | 14 | 0.33 ± 0.18 | 0.17 ± 0.08 | 2.11 ± 1.43 | 15 | 6.72 ± 2.44 | 1.14 ± 0.42 | 6.47 ± 2.56 |
| | M | 14 | 0.25 ± 0.10 | 0.21 ± 0.08 | 1.35 ± 0.68 | 15 | 6.38 ± 1.64 | 1.22 ± 0.52 | 6.43 ± 4.38 |
| S4 | F | 19 | 0.25 ± 0.12 | 0.18 ± 0.08 | 1.89 ± 1.91 | 19 | 6.65 ± 1.82 | 1.29 ± 0.47 | 5.75 ± 2.81 |
| | M | 10 | 0.27 ± 0.14 | 0.20 ± 0.06 | 1.49 ± 0.84 | 9 | 8.89 ± 2.86 | 1.80 ± 0.87 | 5.50 ± 1.86 |

*E. acutus*

| Station | Gender | Body Wall | | | | Gonads | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | n | [RNA] | [DNA] | [RNA]/[DNA] | n | [RNA] | [DNA] | [RNA]/[DNA] |
| S1 | F | 4 | 0.13 ± 0.04 | 0.13 ± 0.10 | 0.93 ± 0.63 | 1 | 1.01 | 11.25 | 11.13 |
| | M | 4 | 0.13 ± 0.10 | 0.16 ± 0.98 | 1.11 ± 0.95 | 0 | - | - | - |
| S2 | F | 10 | 0.15 ± 0.04 | 0.12 ± 0.08 | 0.83 ± 0.55 | 8 | 1.15 ± 0.52 | 5.71 ± 1.51 | 5.94 ± 3.21 |
| | M | 4 | 0.18 ± 0.05 | 0.13 ± 0.05 | 0.70 ± 0.29 | 0 | - | - | - |
| S3 | F | 2 | 0.08 ± 0.01 | 0.04 ± 0.03 | 0.47 ± 0.36 | 8 | 1.94 ± 0.72 | 7.56 ± 2.77 | 4.46 ± 2.38 |
| | M | 5 | 0.18 ± 0.04 | 0.11 ± 0.05 | 0.61 ± 0.26 | 1 | 2.86 | 11.31 | 3.95 |
| S4 | F | 2 | 0.20 ± 0.03 | 0.04 ± 0.03 | 0.21 ± 0.14 | 6 | 1.45 ± 0.61 | 7.99 ± 4.06 | 6.26 ± 3.30 |
| | M | 2 | 0.24 ± 0.01 | 0.12 ± 0.02 | 0.48 ± 0.07 | 0 | - | - | - |

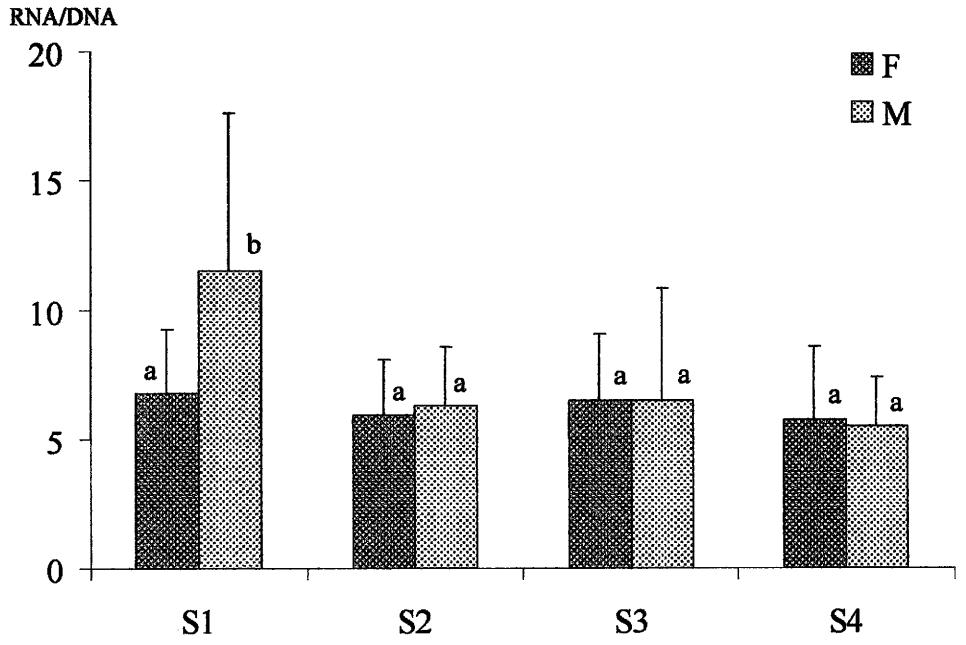


Fig. 5. RNA/DNA ratios determined based on pyloric caeca samples (mean ± standard deviation) from *A. rubens*, for each station (the stations that share the same letter (a-b) do not present a significant difference between their ratios, Tukey tests, $p>0.05$).

## 4. Discussion

### 4.1 Metals contamination

Metal concentrations in sediments of the Sørfjord showed the existence of a gradient as previously reported (Temara et al., 1998; Coteur et al., 2003c). Concentrations in sediments of S1 and S4 differed strongly while the relative concentrations in sediments of S2, S3 and S3II depended on the considered metal. Cadmium (Cd) and zinc (Zn) presented a very clear gradient from S1 to S4. Lead (Pb) showed a clear cut difference between S1 and S2 on the one hand, and the other stations on the other hand. The case of copper (Cu) is more irregular: the S3 station presented the highest concentrations in this element. This station was located in a shore recently banked with rocks from unknown origin. These could have been possibly contaminated with Cu. Despite, these high levels of Cu in sediments, *Asterias rubens* individuals collected in S3 showed the lowest levels of copper in all the body compartments. Coteur et al. (2003c) had proposed that Cu would be tightly controlled in *A. rubens* pyloric caeca and body wall. So, even though this station showed a high sediment contamination of copper, this metal was either not bioavailable or tightly controlled by the starfish.

Comparing to values reported for 2000 (Coteur et al., 2003c) and 2003 (Gillan et al., 2005), it can be seen that sediment contamination has decreased, especially lead levels that, for instance, in S1 were reduced from around 670$\mu g.g^{-1}$ DW (2000), to 260$\mu g.g^{-1}$ DW (2003) and to 58$\mu g.g^{-1}$ DW (2006). In fact, the *A. rubens* body wall contamination by lead also seems to have decreased, comparing to the values reported for 2000 (Coteur et al., 2003c). This metal is not efficiently controlled in this body compartment of starfish (Coteur et al., 2003c), reflecting directly bioavailable levels in the environment. These facts show that remedial actions have been having a positive impact on the quality of the fjord.

It has been reported, for both starfish and sea urchins, that heavy metal accumulation varies according to the body compartment considered and while calcified compartments, like body wall, are regarded as long-term bioindicators, the non-calcified ones are generally considered as short-term indicators (see e.g. Flammang et al., 1997; Temara et al., 1997c; Warnau et al. 1998; Coteur et al., 2003c). In what concerns *A. rubens*, regulation mechanisms were reported for some metals. For instance, cadmium

and zinc are controlled in the pyloric caeca, but not in the body wall (Coteur et al., 2003c). The results presented in this study are in accordance with this regulation model. Contamination levels of zinc and cadmium in pyloric caeca did not follow the gradient established by the sediments. On the contrary, body wall levels are more in accordance with the environmental gradients. The differences observed between genders, in what concerned metal accumulation in starfish gonads, may be due to differential metabolic requisites of male and female gametogenesis.

In sea urchins, the digestive wall is usually the most effective metal concentrator (Warnau et al., 1998 and references there in; Aspholm and Hylland, 1998). In fact, this was observed in the present study except for Pb. Zinc seems to be tightly controlled; there were no differences between concentrations observed from different stations in the gut and in the gonads. Furthermore, the concentrations in the body wall did not follow the sediment contamination gradient.

According to the MDS analysis, that was done using the metal concentrations from the different body compartments, it was possible to group samples from the same stations into distinct clusters. In what concerns *A. rubens*, it was even possible to distinguish two groups composed mainly by male or female individuals. This was probably due to differences in gonads concentrations, already indicated by the ANOVA results. The interpretation of the distance between the samples has to be seen in a relative way. However, it may be concluded that in general individuals from one station are more similar between themselves than with the ones from others, showing that the contamination pattern in each station is distinctive.

### 4.2 Sperm motility

Male gonads of *A. rubens* from S1 and S2 showed significantly higher levels of Cd, Cu, Pb and Zn. However, no effects on velocity parameters of sperm motility, assessed by CASA system, were observed. Similarly, Dubois et al. (2004) observed that, in the same populations, neither fertilization rates nor egg size and morphology were affected. This suggests that *A. rubens* is able to reproduce in environments heavily contaminated by metals. Such conclusion should be further supported by following the embryo and larval developments of the zygotes. Indeed, Guillou et al. (2000) reported high rates of blockage and longest delay in embryonic development of the echinoid *Sphaerechinus granularis* linked to field contamination of the progenitors by iron,

copper, lead, cadmium and mercury. It should be noted that, in the latter case, contamination by organic compounds cannot be ruled out.

### 4.3 RNA/DNA ratio

The RNA/DNA ratio has been extensively used as a biochemical growth rate indicator, providing an estimation of recent growth and metabolic status in a variety of marine organisms (Liyana-Pathirana et al., 2002; Meesters et al. 2002; Melzner et al., 2005; Lannig et al., 2006). Exposure of fish and invertebrates to stress factors (hypoxia and toxicants, like metals), both in laboratory and field conditions, reduced this ratio due to a decrease in RNA content (Wang and Stickle, 1988; Wo et al., 1999; Zhou et al., 2001; Adham, 2002). However, Lannig et al. (2006) reported an increase of RNA/DNA in oysters exposed, in laboratory conditions, to cadmium. This was attributed to the fact that these animals produced proteins associated with metal detoxification. Additionally, the production was temperature dependent, leading to the conclusion that in field conditions the RNA/DNA ratio may be affected by a number of environmental variables, sometimes difficult to predict.

In our results, this ratio did not show any significant differences in the body wall of both *A. rubens* and *Echinus acutus* from different stations. This could be due to the lower metabolism of this compartment compared to digestive organs, for instance.

Male starfish from S1 showed a significantly higher RNA/DNA ratio. This could be linked to the synthesis of detoxification proteins. Indeed, higher metallothionein concentrations were reported in pyloric caeca of *A. rubens* from S1 (Temara et al., 1997a). However, this difference in RNA/DNA ratio was dependent on a few samples (4 among 101). When these data are removed, no significant effect is left. This is further supported by the absence of relation between Cd, Pb, and Cu levels in pyloric caeca and RNA/DNA ratio. Actually, the latter is only related, negatively, to Zn levels in pyloric caeca, an essential metal in metalloenzymes, showing no gradient trend in the pyloric caeca. These facts strongly suggest that RNA/DNA ratio in this starfish of the Sørfjord is not affected by metal field contamination.

## 5. Final remarks

Even though remedial measures have been taken and the quality of the Sørfjord is improving, this fjord still remains as one of the most contaminated places, by heavy metals, in Europe. However, populations of *A. rubens* and *E. acutus* seem to be able to survive under these conditions. These populations of the starfish *A. rubens* have been subjected to several studies during these last few years and although cellular immunity (Coteur et al., 2003c) and metallothionein content (Temara et al., 1997a) were affected by metal contamination, reproduction success (Dubois et al., 2004; present study) and growth (present study) do not seem to be affected. Thus, *A. rubens* appears as a rather resistant species to metals contamination.

This also shows that some biomarkers tend to be more sensitive than others, that metal contamination impacts seem to selectively affect different physiological functions and, therefore, their selection when assessing environmental changes should be carefully done.

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## FINAL CONSIDERATIONS

This study aimed to study the effects of heavy metal contamination using two different biomarkers: sperm motility in the starfish *Asterias rubens* and RNA/DNA ratio in both *A. rubens* and in the sea urchin *Echinus acutus*. The starfish *A. rubens* is considered a valid bioindicator of sediment metal contamination of the Sørfjord, as it readily accumulates metals, although different body compartments are subject to different control mechanisms, depending also on the metal in question (Temara et al., 1998; Coteur et al., 2003b). This was also observed in our study, both for *A. rubens* and *E. acutus*: metal concentrations varied according to the body compartment and to the metal under consideration. For both species, body wall seems to better reflect long term contamination, while the non-calcified compartments seem to reveal short-term changes.

Concentrations in the body wall of starfish populations of the Sørfjord seem to have been reduced comparing to values reported in previous studies (Coteur et al., 2003b). According to the OSPAR Commission (2000), there has also been a downward trend registered in metal concentrations of local populations of blue mussels, although values are still above the background reference concentration (BRC) and their consumption is not advised. In accordance to this, sediment contaminations also seem to be decreasing. This shows, a clear tendency of an improvement on the environmental conditions of the Sørfjord.

It is known that these populations of *A. rubens* were physiologically affected in the most polluted areas of the Sørfjord: metallothioneins concentrations were significantly higher, the alkaline phosphatase activity was reduced (Temara et al.,1997) and cellular immunity affected (Coteur et al., 2003a). However, reproduction success (Dubois et al., 2004; present study) and growth (present study) do not seem to be affected and these populations seem to be able to survive and reproduce. The populations of *E. acutus* also seem to be well established and body wall growth did not seem to be affected by metal contamination. This shows that some biomarkers tend to be more sensitive than others and that metal contamination impacts seem to selectively affect different physiological functions, and, therefore, their choice to assess environmental alterations should be carefully done.

The selection of an adequate biomarker for a certain period of time is a key-issue in ecotoxicology. Therefore, it can be concluded that field studies and biomonitoring programmes seem to be important in establishing the environmental status of this fjord and in analysing the relationships with bioindicator species and the contamination levels of their environment. The Sørfjord has been included in several pollution monitoring programmes, including *The Norwegian State Pollution Monitoring Programme* (NSPMP) and the *International Joint Assessment and Monitoring Programme* (JAMP) administrated by the Oslo-Paris Convention (OSPAR) (see Ruus et al., 2006, and references therein). The information obtained in these programmes should be used in order to develop ecotoxilogical studies in the future, that will contribute to a better and more integrated knowledge on the effects of contaminants in biological communities.

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