SPSD II

EFFECTS OF POLLUTANTS ON BENTHIC POPULATIONS AND COMMUNITIES OF NORTH SEA ORGANISMS "ECOTOX2"

PH. DUBOIS, R. BLUST, R. FLAMMANG, E. DE PAUW, M. JANGOUX

PART 2

GLOBAL CHANGE, ECOSYSTEMS AND BIODIVERSITY

ATMOSPHERE AND CLIMATE

MARINE ECOSYSTEMS AND BIODIVERSITY

TERRESTRIAL ECOSYSTEMS AND BIODIVERSITY

NORTH SEA

ANTARCTICA

BIODIVERSITY

SCIENTIFIC SUPPORT PLAN FOR A SUSTAINABLE DEVELOPMENT POLICY

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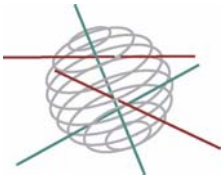
(SPSD II)



Part 2:
Global change, Ecosystems and Biodiversity – North Sea

FINAL REPORT

EFFECTS OF POLLUTANTS ON BENTHIC POPULATIONS AND COMMUNITIES OF NORTH SEA ORGANISMS

"ECOTOX2"

EV/23

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ABSTRACT

The goal of the present project was to determine if the effects of contaminants of high concern in the North Sea result in significant impairments of populations or communities of benthic organisms in the North Sea and if the early signals provided by biomarkers can effectively predict these ecological effects.

Effects of metals and dioxin-like compounds on natural populations of starfish *Asterias rubens* were studied. Gamete quality, larval survival, juvenile growth and RNA/DNA ratios in adults were investigated. No effect could be unequivocally attributed to contaminants, especially in postmetamorphic stages. However, embryo production and larval survival are clearly were affected with some population producing no viable larvae on some year. This deleterious effect is probably compensated at the population level by the large dispersal capacity of the studied species.

Trends in metal contamination of mussels *Mytilus edulis* were investigated in populations from the Westerchelde. A clear drop of metals in mussels was observed in the early 80s coinciding with the start of the efforts to reduce chemical pollution input into the North Sea. Since those early reductions, metal concentrations in mussels generally remained unchanged up to mid 90s. However, in recent times metal concentration in mussels have increased significantly, for example Cd in 2002 was almost 10 times the values in 1983 and similar to levels seen during the peak in the 70s. It is suggested these increased metal concentrations are a result of changes in physical and chemical speciation and metal bioavailability.

Analysis of sediment-associated bacterial communities (SAMC) showed that these communities respond to metals and are possibly able to adapt to high contamination levels.

It is concluded that taking into account effects at the individual level for risk assessment policies is relevant and should be on the safe side when considering possible effects at the population level.

INTRODUCTION

The Quality Status Report 2000 of the OSPAR Commission for the Greater North Sea (QSR 2000) underlines that concentrations in the environment and biota of most of the "trace organic contaminants" and "heavy metals" ranked in "Priority classes of human pressures" A and B (i.e. the two classes of human pressures with the highest impact) show, when known, absent, or only slow decreasing trends. Furthermore, the QSR 2000 emphasizes that most of these contaminants present either generally or locally high levels. These worrying contaminants include coplanar polychlorobiphenyls (cPCBs), dioxins, furans, polycyclic aromatic hydrocarbons (PAHSs) and some metals (Cd, Pb, Hg). Potential risks from other, lesser known, contaminants from diverse sources, namely polybrominated diphenyls ethers (PBDEs, used as flame retardants as replacement compounds of PCBs) and Zn are also subjects of concern. The QSR 2000 further indicates that "the chronic and combined effects that [these] hazardous substances have on organisms are not well known, which seriously hampers the assessment of their environmental risk". In particular, it states that endocrine disruptors (like cPCBs, dioxins, furans, PBDEs, and PAHSs) are "one of the issues of emerging concern". It recommends that "effort should be invested in developing tools for the assessment of substances and effects of concern, taking into account the merits of integrating biological effects and chemical monitoring approaches" and that "further development of biomarker techniques […] is crucial".

Current research, including data from the proposing laboratories, indicates that these contaminants are effectively accumulated in benthic biota of the North Sea, some of these at very high level in comparison with terrestrial biota (e.g. cPCBs, dioxins, furans in the mouth of the Westerscheldt River, or metals in river mouths and localized hot spots as the Sørfjord in Norway; Hong & Bush 1990, Temara *et al.* 1998, De Pauw *et al.*, unpubl., Wantier *et al.*, unpubl.). Levels of others (like PBDEs) are almost totally unknown but their potential hazard is high (De Boer *et al.* 1998). Significant effects of some of these compounds (or their mixture) have been detected in several organisms at biochemical to individual levels: induction of detoxification processes (metallothioneins, activation of cytochrome P450 enzymatic activities; Temara *et al.* 1997, Roesijadi *et al.* 1997, den Besten *et al.* 2001), impairment of the immune system (Coles *et al.* 1995, Dyrynda *et al.* 1998, Coteur *et al.* 2001) or larval development (Gosling 1992, Kobayashi 1995, Pagano *et al.* 1996, Gosselin *et al.* unpubl.). Furthermore, the impact of these contaminants appears to be either linked or emphasized by increased levels of organic matter in the environment (possibly linked to eutrophication) (Hylland *et al.* 1996, Riveros *et al.* 1996). These effects were documented at their own level of biological integration (*i.e.* at most at the individual

level) but there are few data to interpret the significance of these effects on population and communities, especially in benthic invertebrates (reviewed by Kennish 1998). This is particularly important in the case of effects at the biochemical level that can be used (and sometimes are already used) as biomarkers of exposition (i.e. giving early signals of contamination).

Effects at the population level may involve, in adults, reduced and/or differential survival (some age groups surviving better), reduced growth rate, and reduced fecundity (quantity and/or quality of gametes produced). Benthic organisms with a planktonic larval stage will further depend on larval supply (and thus on larval survival), successfull substrate selection and metamorphosis, and early juvenile survival (Cameron & Schroeter 1980). Effects at the community level may encompass biodiversity reduction, community shifts (change in dominant species), and alterations of trophic relationships. Effects of the respective contaminants and increased organic charge are differently investigated according to the life stage (adult, larva, metamorphic) and substance considered. In adult benthic invertebrates, effects of inorganic metals on growth rate are rather well-known in bivalves and crustaceans as well as effects on biodiversity of communities. Cadmium has also been reported to affect bacterial biomass (Fabiano *et al.* 1994). Effects on population dynamics are, however, poorly documented (Guillou *et al.* 2000). Effects of halogenated hydrocarbons at population level are mainly documented in vertebrates with very few data on benthic invertebrates (see Kennish 1998). Increased organic enrichment has been linked to reduced biodiversity and growth, and to shifts in community assemblages (e.g. Weston 1990).

In planktonic larvae of benthic invertebrates, effects of metals and to a lower extent of PCBs are rather well-known for the endotrophic phase but poorly investigated for the exotrophic phase. Effects of other contaminants (dioxins, furans, PBDEs, PAHS) are almost uninvestigated (see e.g. Kobayashi 1995, for a review). Effects of food quality (mainly microalgae) on larval developement has been documented (Strathmann 1971, Pedrotti & Fenaux 1993, Boidron-Métairon 1995, Basch & Pearse 1996, Vaitilingon *et al.* 2001). However, the food value of microalgae favoured by eutrophication (*Phaeocystis spp.*, *Rhizosolenia spp.*; Rousseau 2000) is uninvestigated. Furthermore recent reports indicate that some microalgae may prove embryotoxic (Aanesen *et al.* 1998, Buttano *et al.* 1999, Miralto *et al.* 1999). Metamorphosis of indirect developing animals implies tremendous reorganization of the body. This reorganization is under control of numerous factors, including endocrine factors (Chen & Huang 1990, Chino *et al.* 1994, Yazaki & Harashima 1994). There is thus a serious risk that contaminants, in particular endocrine disruptors will affect these processes. Metals were shown to affect metamorphosis in the course of SPSD1 (Gosselin *et al.* unpubl). To our knowledge, there are no data available on the toxicity of other xenobiotics on this important event.

Accordingly, the goal of the present project was to determine if the effects of contaminants of high concern in the North Sea result in significant impairments of populations or communities of benthic organisms in the North Sea and if the early signals provided by biomarkers can effectively predict these ecological effects.

Considered biota were chosen according to their ecological and/or strategic importance. Some organisms were studied at the population level, others at the community level. At the population level, two species were studied: the starfish *Asterias rubens* and the mussel *Mytilus edulis*. These species can be considered to a certain point as resistant to pollution, they are established or proposed bioindicators of pollution, they belong to two successive steps of the same trophic chain and both are dominant or key-species of the ecosystems in which they occur (Menge *et al.* 1994, Navarrete & Menge 1996, Everaarts *et al.* 1998, Temara *et al.* 1998, den Besten *et al.* 2001). Furthermore, echinoderm larvae (including those of *A.rubens*) were demonstrated to have been dominating the mesozooplankton from the 1980s (Lindley *et al.* 1995). At the community level, sediment-associated microbial communities were studied. They make a rather poorly studied benthic compartment despite their ubiquity and tremendous ecological importance (10^6 to 10^9 bacteria occur per gram of sediment) (Austin 1988, Brock *et al.* 1994). Their communities apparently respond very quickly to contamination thanks to their short generation time and include species very resistant to pollutants as well as species very sensitive (Ford 1994).

MATERIALS AND METHODS

1. Echinoderms

1.1. Gamete quality

1.1.1. Study Area and Sampling

Sampling took place in the Sørfjord, which is 37 km long, l to 2 km wide and 390 m in maximum depth. Starfish *A.rubens* were collected in March 2006 by SCUBA diving in five different stations at increasing distances from the fjord head: S1, S2, S3 and S4 (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 Jåstad, Aga. Samples, three per station, of the top 5 cm layer of sediments were collected in polyethylene containers. They were stored at -20ºC and then oven dried (60ºC for 2 d) before metal analysis. A sub-sample was dry-sieved in order to separate the less than 63 µm size fraction.

Table 1. Location and conditions of the different sampling sites in the Sørfjord, Norway.

Site	Coordinates		Distance from the head of the fjord (km)	Collection Depths (m)	Water Temperature (ºC)	Salinity at sampling depth (psu)
S1	60º04.79 N	06º31.81 E	1.6	1.5-9.0	5-9	33-38
S2	60º09.02 N	06º32.76 E	9.0	3.0-6.0	5-8	34-36
S3	60º12.58 N	06º33.80 E	15.5	2.5-5.0	7	34-35
S3 II	60º14.61 N	06º33.80 E	19.5	8.0	6	35
S4	60º24.93 N	06º31.55 E	39.6	2.0-6.0	4-6	35-36

To assess egg size and morphology as well as fertilization success, starfish of 50 to 70mm ray length (measured from the mouth to the arm tip) were collected in the four sites between 2 and 8m depth by SCUBA divers between March 10th and 13th, 2003. At that time, no vertical salinity gradient was apparent in the first 10m (salinity ranged between 29.5 and 31.6 ppt according to the sampling site). Starfish were brought back to the laboratory in Odda in the water of the site and immediately processed.

1.1.2. Metal analysis

Cd, Cu, and Pb were analysed by graphite-furnace atomic absorption spectrometry (GF-AAS) and Zn by Flame atomic absorption spectrometry (F-AAS) as described in Coteur et al. (2003b).

1.1.3. Egg size and morphology; fertilization success

Starfish were sexed by collecting some gametes through the body wall with a syringe fitted with a needle. Eleven to sixteen females were induced to spawn in filtered (0.22µm) sea water of the site by injecting 0.2ml of 1-methyladenine, 1 µM in Milli-Q water, into the general coelomic cavity of each arm. Spawning was expected to occur within 1h of injection. Seven to eight females in each population sample were successfully induced to spawn. After spawning, eggs were collected on a microscopic slide and photographed with a Q-Imaging Micro-Publisher digital camera (3.2 Mpixels) mounted on a Leitz Laborlux D optical microscope. Egg size was assessed by measuring their area on micrographs. Egg morphology was assessed by the ratio between the largest and smallest diameter of each egg (a circularity index). Areas and largest and smallest diameters were measured using the image analysis program ImageJ 1.29 running under MacOS10.2. Three hundred eggs were measured for each female. The calibration of digital measures was carried out by photographing a stage micrometer with the same device. Images were converted to 8-bit and background was substracted before particle analysis was carried out using the software routine using a double grey threshold (44 and 232). Thresholding and particle selection was checked by eye for each image and manually corrected if necessary.

After spawning of the females, males were induced to spawn in the same way. A pool of sperm from three males from the same site was added to concentrated eggs within 45min of its emission. After 1h, fertilization success (elevation of fertilization membrane) was assessed using a dissecting microscope.

1.1.4. Sperm Motility

For sperm motility analysis, 20 *A. rubens* individuals were collected from each station and brought to Belgium, in aerated water, no later than 3 d after their collection. They were then installed in a closed circuit aquarium system (sea water: 35 psu at 10ºC; animals were not fed) and within two weeks sperm motility video recordings were carried out.

Asterias rubens male individuals were identified by collecting some gametes through the body wall with the help of a syringe and then placed in individual aquaria containing a small amount of filtered (0.22 µm) sea water from S4 at 35 psu (1 cm layer). Spawning was induced by injecting in the general cavity of each arm 0.2 to 0.4 mL of 1 µM 1-methyladenine solution. Spawning took place from 1 h 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, 5 µL of dry sperm were diluted in 8 mL of filtered sea water, previously collected at S4, and gently mixed. Sub-samples of 150 µL were immediately mounted on a glass slide chamber. Sperm movement was recorded, during a maximum of 10 s, at room temperature, with a CCD (charge-coupled device) camera (Fire-i™ 400™ Sony©, San Diego, CA, USA) attached to a microscope, in dark-field mode. Video image scale was calibrated using a micrometer slide.

Furthermore, a sample (50 µL) of diluted sperm was placed in 2450 µL of 5% formol solution and then number of spermatozoa in each sample was assessed with the help of a haemocytometer (0.0025 mm^2).

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: curvilinear velocity (VCL), the time average velocity of the sperm head along its actual trajectory; 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; 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 µm s^{-1}.

The number of males used per station was four for S1, four for S2, three for S3 and one 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 five and 12 (derived from two sub-samples) per male, being usually eight, and making a total of 96, viz. 29 video recordings for S1, 36 for S2, 23 for S3 and eight for S4.

1.2.Larval survival

1.2.1. Study area

This study was carried out in 5 locations over a relatively broad geographical range (Scharendijke, Knokke, Ambleteuse, Aber and Piriac respectively from the Netherlands to south Brittany, France). All sites were intertidal except Scharendijke. They represent a wide range of environmental situations (Table 2) and a large panel of contamination.

Table 2: Characterization of the studied locations and population reproductive cycles. Maximal annual salinity ranges were established from measurements at low tide in intertidal locations and at 2 meters depth in the subtidal location. LTWL: Low Tide Water Level (m) at which the intertidal populations are totally emerged. Age at which first maturity size was reached was deduced from temporal evolution of the mean size of cohorts (delimited by modal analysis using maximum likelihood); it was expressed in month after recruitment from Joly et al. sunpublished .

Locations	Piriac	Aber	Ambleteuse	Knokke	Scharendijke
	48°24' N 2°31' W	48°14' N 4°27' W	50°44' N 1°30' E	51°21' N 3°18' E	51°45' N 3°51' E
Maximal annual temperature range in °C (month of occurrence)	9 (Jan) 20 (Aug.)	9 (Jan.) 21 (Aug.)	6 (Jan.) 20 (Aug.)	5 (Jan.) 20 (Aug.)	6 (Jan.) 18 (Aug.)
Maximal annual salinity range (PSU)	30-34	32-35	31-35	28-35	27-33
LTWL (m)	Connection with the subtidal area			1.8	Subtidal
Community	Mussel beds on natural rocks	Pebbles accumulation	Mussel beds on natural rocks	Mussel beds on a breakwater	Shellfish beds, mud and rocks on mud
Age at maturity size (months after recruitment) *Year of first reproduction*	5 to 8 months *2nd year*	Unknown *Unknown*	4 months *1st year*	7 to 9 months *2nd year*	Unknown *Unknown*
Spawning period	April	May-June	May-?	April-May	April-June

1.2.2. Heavy metals contamination of the populations

In April 2003, the level of heavy metal contamination in the different populations was determined in 2 compartments, gonad and pyloric caeca, from 10 starfish of each population. Fresh starfish of 50-70 mm ray length were collected and immediately dissected in the field. They were sexed by microscopic observation of a smear. Gonads and pyloric caeca were removed and frozen at -20°C. Heavy metal analysis was carried out as described in Coteur et al (2003b).

A principal component factor analysis was performed with varimax rotation on heavy metal concentrations in each body compartment as variables (n = 8). Accepted factors had an eigenvalue superior or equal to 1.0. A 2 factor ANOVA (factor "location" and "sex") was performed for each factor on the factor scores.

1.2.3. Embryo and larval development: general procedures

Starfish of 50-60 mm ray length were collected by hand at low tide in the intertidal populations and by scuba-diving in Scharendijke. Concomitantly, sea water was collected in each site. Before fertilisation assays, the starfish were maintained in

aquarium at the field temperature for no longer than 3 days. The sea water (SW) was filtered (25 µm glass fiber) and kept in aerated flasks at 14°C in the dark. A sub-sample of 2 litres of each sea water was filtered on 0.22 µm and kept under the same conditions.

1.2.3.1. Spawning induction

Starfish were sexed by puncturing gonadic material through the body wall with a syringe. Starfish were induced to spawn with 1-Methyladenine 100µM in sea water (1ml per 100gFW, equally injected in each arm) in 1L aquaria containing 25 µm-filtered SW (Coteur et al. 2003a, Kanatani & Shinai 1972). As sperm fertility decreases faster after spawning than egg fertility, females were first induced to spawn (Williams & Bentley 2002). Females who did not spawn within 75 minutes after injection were discarded. Gametes were collected by pipetting. Sperm of the 3 first males to spawn were pooled. Eggs and sperm were stored at 14°C for no longer than, respectively 3h and 50 mn after emission.

1.2.3.2. Fertilization

Eggs were spread in 90mm Petri dishes so that a homogenous single layer of eggs was obtained. A few drops of the sperm pool was added in each Petri dish (Coteur et al. 2003a). When fertilization was successful (presence of fertilization membrane), zygotes were distributed in sterile 6-well plates (about 200 zygotes per well in 10 ml of 0.22 µm filtered sea-water). Plates were kept in the dark at 14°C.

1.2.3.3. Counting of larval type

After 72 hours, i.e. at the end of the endotrophic stage, 1ml of 40% formaldehyde was added to each well to kill and preserve the larvae and plates were heated at 60°C over night (Spirlet, com. pers.). The different types of larvae (dead, normal and viable larvae) were counted in each well (100 larvae minimum counted per well). Normal larvae are kidney-shaped, presenting a complete archenteron and pairs of coelomic pouches (Coteur et al. 2003a). Retarded larvae are smaller with an incomplete archenteron but do not show abnormalities. Abnormal larvae show skeletal or gut malformations or are blocked at the blastula or gastrula stages. Viable larvae correspond to normal plus retarded larvae. A first count allowed us to assess the percentage of dead larvae, while a second count which excluded them, was used to assess the proportion of the other larval types.

1.2.3. Larval viability in natural populations

Assays were carried out in 2002 and 2003 during the spawning periods of the studied populations: in the North Sea in April 2002 (Scharendijke) and in March and April 2003 (Ambleteuse, Knokke and Scharendijke), in Brittany in April and May 2002

and 2003 (Aber and Piriac). Spawning, fertilization and embryo-larval development were always carried out in the sea water collected in the location from which the genitors originated at its normal salinity.
In this experiment, eggs of each female (n=8 to 16) from one location were assayed separately using a pool of sperm from 3 males of the same location. No selection of the gametes was carried out. Four replicated wells per treatment were used.

1.3. Embryo assays

1.3.1. General protocol of the embryotoxicity assays

A standard protocol of embryotoxicity assay was developed in our laboratory (Coteur *et al.* 2003c). These embryotoxicity assays were centered on the endotrophic larval development of two echinoderm species: *Psammechinus miliaris* (Echinoidea) and *Asterias rubens* (Asteroidea). This sequence of the ontogenic development, running from the early gastrula stage to the end of the endotrophic larval stage, lasts about 3 days for the considered species. It implies the development of the digestive tract and of most of the basic larval structures (ciliated band, larval arms, etc.). In Echinoidea, comparative studies have already demonstrated that this part of the life cycle is very sensitive to natural and anthropogenic contaminants (Kobayashi 1995). Embryotoxicity assays based on Asteroidea development are not so well documented but appear as very promising (Den Besten 1989). Assays were directed on species from two different Echinoderm taxa in order to increase the ecological significance of our tests. The Asteroidea *Asterias rubens* is important because it is a key species of the benthic fauna along the Belgian coast. The Echinoidea *Psammechinus miliaris* is not as abundant as *A. rubens* in the Belgian area from the North Sea but is one of the two European sea urchins the most used in ecotoxicological studies (Sheridan & Massin 1998). During the project ECOTOXII, the protocol of our bioassay was adapted to quantify the toxic effects of two main sources of pollution in the North Sea area: the Polycyclic Aromatic Hydrocarbons (PAHs) and the recurrent blooms of the microalga *Phaeocystis globosa*.

Bioassays were performed using natural sea-water collected in front of the marine station of Luc-sur-Mer (Normandy, France). Sea-water was previously allowed to decant for 48 h, filtered by a 10 µm membrane and maintained at 20 ± 1°C (*i.e.* FSW).

Adult genitors were collected intertidally in France (*P. miliaris* at Luc-sur-Mer and *A. rubens* at Wimereux). Individuals were transferred in the cultivation system of the marine laboratory of Mons. Spawning was induced by injection into the coelomic cavity of KCl 0.5 N (20 µl per gram; *P. miliaris*) or 1-methyl-adenine 100 µM (1 ml for each estimated 100 ml in body volume; *A. rubens*). Embryos at early gastrula stage (4-5 h after fertilization at room temperature) were used for experiments.

Bioassays were performed in six-well plates (Falcon, ref. 35-3046). Batches of about 250 embryos at early gastrula stage were transferred in each well previously filled with 10 ml of FSW including or not some contaminant. After 72 h at 14±1°C, the frequency of developmental defects was scored in each well on a random sample of 100 larvae. Developmental defects were scored according to the morphological criteria adapted from Warnau & Pagano (1994). Larvae were classified as (1) Normal (N), (2) Delayed (D), (3) Highly delayed (H), or (4) Abnormal (A). Abnormal stage corresponded to Pathologic 1 stage of Warnau & Pagano (1994). Table 3 summarizes the main characters of each of our "morphotypes" (viz. morphological types). Complementary test were performed on "Viable" larvae. The rates of "Viable" larvae corresponded to the sum of the rates of "Normal" and "Delayed" larvae in each replicate.

1.3.2. Toxicity of Polycyclic Aromatic Hydrocarbons

Embryos were exposed to a range of concentrations of single PAH either in complete darkness or under ultraviolet radiation. Ultra-violet light was provided by one "Cosmolux UVA plus 80-W" lamp. Larvae were exposed to the UV radiation during all the duration of the experiment (72 h). Our tests implied seven PAHs including a growing number of benzene nucleus. Table 4 resumes the main physico-chemical properties of those seven PAHs. The phototoxic properties of benzo[α]pyrene are well documented (see review by Lyons *et al.* 2002). PAHs were inoculated at the final concentration of 0.01, 0.1, 0.5, 1, 5, 10, and 100 µg/l. Control experiments implied larval developments performed in FSW and in FSW previously inoculated with the same dose of acetone than the experimental series. Each type of control experiment was performed either in darkness or under UV radiation.

Table 3. Larval morphotypes of *Psammechinus miliaris* and *Asterias rubens* at the end of embryotoxicity tests.

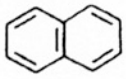
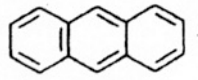
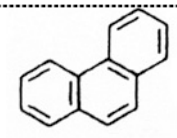
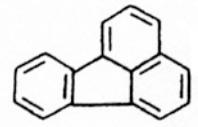
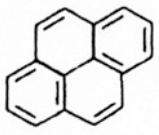
Type	Abbre-viation	Description in	
		Psammechinus miliaris	***Asterias rubens***
Normal	N	Pyramidal larva with a differentiated gut, a first pair of coeloms, a pair of trifid larval rods and a discrete net of intra-blastocoelian mesenchymatous cells.	Pear-shaped larva with a differentiated gut, a first pair of coeloms, a discrete net of mesenchymatous cells in the most anterior part of the blastocoel.
Delayed	D	Larva similar in dimension to normal ones and presenting an incomplete organogenesis though deprived of morphological abnormalities.	

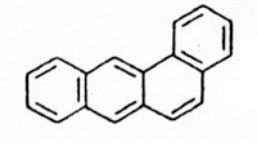
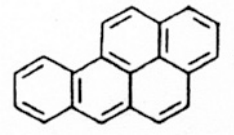
Highly delayed	H	Larva smaller than normal ones and presenting an incomplete organogenesis though deprived of morphological abnormalities.
Abnormal	A	Larva presenting external or internal morphological abnormalities.

1.3.3. Toxicity of the Phaeocystis colonies and derived products

To assess *P. globosa* toxicity, developing larvae of an asteroid (*Asterias rubens*) and an echinoid (*Psammechinus miliaris*) were exposed to water from the Southern Bight (*i.e.* water collected after a bloom of *P. globosa*) and to substances produced by laboratory cultivated *P. globosa*. For comparison, diatom-derived substances were obtained from cultures of *Skeletonema costatum.*

Table 4. Structure and properties of the seven PAHs studied (Napgal 1993).

PAH (International Abbreviation)	Formula	Number of benzene nucleus	Molecular weight	Solubility in water at 25°C (µg/l)	Log Kow	Carcino-genicity
Naphtalene (NA)	 C10H8	2	128.2	12,500<34,000	3.37	-
Anthracene (ANT)	 C14H10	3	178.2	59	4.5	-
Phenanthrene (PH)	 C14H10	3	178.2	435	4.46	-
Fluoranthene (FLAN)	 C16H10	3	202.3	260	4.9	-
Pyrene (PYR)	 C16H10	4	202.3	133	4.88	-

Benzo[α]-anthracene (B[a]A)	 C18H12	4	228.3	11	5.63	+
Benzo[α]-pyrene (B[a]P)	 C20H12	5	252.3	3.8	6.21	+++

1.3.3.1. Field sampling of sea water

During the March 2004 Belgica cruise, samples of sea water (Belgica sea water; BSW) were collected in the Southern Bight using Niskin bottles at stations 250 (51°30.02N-03°19.25E) and VB8 (51°23.44N-2°40.35E). Sampling occurred just after a natural bloom of *P. globosa*. Samples were stored at 14±1°C for less than 3 days. They were filtered using 0.2 µm Sartorius membrane and sterilized 20 min at 121°C before to be used for experiments.

Dilutions of the BSW (and of BSW organic extracts; see below) were performed using FSW (*i.e.* natural sea water collected in front of the marine station of Luc-sur-Mer (Normandy, France) that was previously allowed to decant for 48 h, filtered on a 10 µm membrane and maintained at 20 ± 1°C).

1.3.3.2. algal cultures

Cultures of *Phaeocystis globosa* were prepared according to the procedure of Hansen *et al.* (2003). Algae were obtained from samples of the strain BCZ99 originating from station 330 of the Belgian coastal zone. Artificial sea water (ASW) for algal cultures was prepared according to the method of Harrison *et al.* (1980), and nutriments were added following Veldhuis & Admiraal formulation (1987). *P. globosa* was cultivated under three different N:P ratios: 5, 16 and 100 (16 being the normal N:P ratio of oceanic waters; Redfield *et al.* 1963). *S. costatum* was cultivated under N:P ratio 16 only.

Cultures were performed in 2 l glass balloons maintained at 10 ± 1°C and exposed to a 14h/10h light/dark cycle (Philips TLD 30W/86). No nutriment was added to the cultures during the growing period. About 10 days were necessary to obtain cultures having 10^6 to 10^7 cells per liter. The water in which algae were cultivated (*i.e.* CSW), as well as the organic extract of CSW, were diluted using ASW.

1.3.3.3. Extraction of organic matter

During their growing period, *P. globosa* released organic substances that are either emitted in the culture water (CSW) or concentrated inside the matrix of the colony. To assess their relative toxicity, two different filtration processes were used to

separate the colonies from the surrounding water: the normal filtration (implying glass microfibre GF/C filters able to retain particles down to 1.2 µm) and the inverted filtration (using the principle of the "*Bodrum*" coffee machine). Normal filtration tears the matrix of the colonies (*i.e.* broken colonies), and the organic compounds of the matrix are thus released in the filtered water. Inverted filtration preserves the integrity of the colonies (*i.e.* undamaged colonies) and allows to concentrate them in a small volume of culture water forming the so-called "*concentrate*". Inverted filtration results in that the organic compounds occurring inside the matrix are kept within it and the filtered CSW (FCSW) only contains the compounds released by the colonies during their growing period. The FCSW, the used GF/C filters and the concentrates were stored in PVC bottles at -80°C.

Extractions of organic matter were performed from colonies collected either on the filters (broken colonies isolated by normal filtration) or in the concentrates (undamaged colonies isolated by inverted filtration). Extractions were also performed from the FCSW. They were carried out using ethyl acetate or methanol as solvent.

* Extractions from the FCSW were performed from pre-conditioned samples of 200 ml to which 200 ml of solvent were added (liquid-liquid extraction). These extractions were repeated three times on the same sample and the four volumes of solvent were eventually combined (final volume of solvent: 800 ml).
* Similar extractions were performed from the concentrates, the latter being each time the result of the inverted filtration of CSW.
* Extractions from broken colonies were carried out directly from the filters (solid-liquid extraction). To work on similar number of colonies, extractions were each time performed from the set of four filters necessary to filter 200 ml of culture water: each filter was put in 200 ml of solvent, then ultrasonicated for 5 min at 4°C and stored one night at 4°C. The amounts of solvent used for the same set of filters were eventually combined (final volume of solvent: 800 ml).

To concentrate the compounds extracted from the CSW and from the colonies, the solvents were totally evaporated using a rotorvapor at low temperature (40°C) and under low pressure. These obtained substances were solubilized in 10 ml of acetone and stored at -80°C before to be used in embryotoxicity assays.

The two different filtration processes and the same protocol of liquid-liquid extraction were also used to isolate the organic matters from samples of BSW collected at stations 250 and VB8

1.3.3.4. Embryotoxicological experiments

Embryos were exposed to ranges of dilutions of FBSW, FCSW and their different related organic extracts. Experimental series performed in FSW and ASW were used as controls. No larval mortality was observed, and larvae mostly reacted

by slowing down their growth during their development. The term "allelopathy" was used to qualify this particular "toxic" effect.

1.4 Juvenile growth

1.4.1. Field study

The study was carried out at 4 intertidal locations chosen to encompass a large range of environmental situations and a relatively broad geographical range, Breskens (the Netherlands), Knokke (Belgium), Ambleteuse (Nord-Pas-de-Calais, France) and Aber (Brittany, France) (Table 2). All of these locations were known to have harboured stable starfish populations from more than 2 decades (Guillou 1979, Dubois, com.pers., 1983, Jangoux & Vloebergh 1973).

Each location was visited monthly. Knokke, Aber and Breskens were first visited in April 2000, Ambleteuse in January 2001. The Breskens population was followed until June 2002 and the 3 others until November 2002. Temperature and salinity were measured at low tide using an electronic thermometer (Ama-digit ad15th) and a hand refractometer (Atago S/Mill). As many starfish as possible were carefully collected at each visit (up to 500). Their ray was assessed by the length of the longer arm from mouth to tip, in millimeters, using Vernier calipers. Regenerating starfish were not considered.

For modal analysis of size frequency distributions, the individuals were pooled in size classes of 5mm range. A sum of normal distributions (called below "cohorts") was adjusted to each monthly size frequency distribution using MIX 3.0 (Macdonald & Pitcher 1979), which is based on the maximum likelihood determination. Samples with less than 150 individuals were discarded from the modal analysis.

In February and August 2001, the area where the recruits first appeared was sampled to determine the abundance and type of food available to juveniles. Three random samples of 40 cm² of rocks were scrapped. They were fixed in formaldehyde (7% in sea water) and then sorted in the laboratory. This was done in each location except Ambleteuse where recruits are detected deep in cracks, which were unsuitable for sampling. The fauna was determined to the genus level and then grouped in taxa for organic biomass determination. Taxon subsamples were first dried at 60°C for 48 h and weighted and then burned at 450°C for 4 h (Majeed 1987) and weighted again allowing the determination of the organic weight.

In Ambleteuse, a recapture study on large starfish (ray > 80 mm) was carried out in September 2001. The extremity of the 5 arms of 150 of them was stained with Nile-blue (Feder 1970). Recapture was carried out one month later.

1.4.2. Sampling for contaminants and analysis

In order to determine the level of metal contamination in the different populations, 10 starfish of 35- 45 mm ray from each location except Breskens, where no individual large enough for analysis was available, were sampled between February and April 2001. They were immediately dissected in the laboratory and processed for heavy metal analysis of the pyloric caeca and body wall following the methods described by Coteur *et al* (2003b).

For dioxin-like compounds, samples were collected between October and November 2001. Sea stars (*A. rubens*) and mussels (*M. edulis*) were collected by hand (seashore fishing) in 5 intertidal stations, along the French and Belgian coasts (Table 5), and immediately frozen (-20°C) in 400 ml polypropylene containers. Only specimens belonging to the same size-class (5-7 cm from the tip of the arm to the centre of the mouth for sea stars and 3-4 cm shell length for mussels) were considered. Sea stars collected for contaminants analysis were dissected and pyloric caeca (digestive and storage organs) were pooled by 5 (3 pools per station) and frozen at -20°C until analysis. Pyloric caeca of 3 individuals per pool were sub-sampled (approx. 1 ml) for CYP1A immunopositive protein (CYP1A IPP) measurements. These sub-samples (n=9) were frozen in liquid nitrogen and transferred to a deep freezer (-80°C). Collected mussels (3 pools per station; ca. 1kg fresh wt with shells per pool) were washed with sea water, and frozen with the shell (-20°C) before analysis.

Table 5: Positions and characteristics of the sampling stations for the analysis of dioxin-like compounds

Station code	Coordinates (N)	(E)	Salinity (‰)
Ambleteuse	50°44.50	1°35.00	33.0
Nieuwpoort	51°08.80	2°42.80	32.5
Oostende	51°13.80	2°54.40	32.0
Wenduine	51° 17.80	3°04.40	32.7
Knokke	51°20.80	3°17.80	32.0

1.4.2.1. PCDD/Fs analysis

All analyses were carried out in the Laboratory of Mass Spectrometry, Centre for the Analysis of Trace Residues (CART), University of Liège (ISO-17025 accreditation, certificat n°212-T), using the method described by Focant et al. (2001).

Briefly, samples were extracted using an Accelerated Solvent Extractor (ASE 200, Dionex), before gravimetricallly determining their lipid content. After dissolving the extracts in hexane/dichloromethane (1:1, 50 ml) fat extracts were spiked with 10 µl of

the internal standard (17 ^{13}C-labeled PCDD/F congeners, EDF-4144, LGC Promotion and 4 ^{13}C-labeled c-PCB congeners, Campro Scientific WP-LCS).

An HCDS column (High Capacity Disposable Silica; 28 g acidic, 16 g basic, 6 g neutral) was added to the classical Power-Prep set of columns (multi-layer silica columns (4 g acid, 2 g base and 1.5 g neutral), basic alumina (8 g) and PX-21 (2 g) carbon column), all manufactured by Fluid Management System (USA). Seventeen PCDD/Fs congeners, and 4 coplanar PCBs (IUPAC Nos. 77, 81, 126, 169) were quantified in the samples using a gas chromatography column coupled to a high resolution mass spectrometer (GC-HRMS, Autospec, Ultima). The mass spectrometer was operated in the electron impact ionization mode using selected ion monitoring (SIM).

Results were expressed either as pg g^{-1} of lipid weight or in terms of toxicity, using WHO human TEF (Van den Berg et al.. 1998) as pg WHO TEQ g^{-1} of lipids weight. Quality controls (QCs) were performed following the EN1948-3 norms, for both samples and blanks (Focant et al. 2001).

1.4.2.2. Cytochrome P450 immunopositive protein (CYP1A IPP) induction

CYP1A IPP induction was quantified using competitive ELISA (Danis et al. 2004). The ELISA was carried out using competition for anti-CYP1A antibodies between the CYP1A immunopositive protein from sea star samples and biotinylated ß-naphtoflavone (BNF)-injected trout (*Oncorhyncus mykiss*) microsomes displaying high concentrations of CYP1A (Biosense, Norway). Multiwell plates were coated with anti-CYP1A (rabbit anti-fish CYP1A peptide, polyclonal antibody purchased from Biosense, Norway). Wells were washed with phosphate-buffered saline (PBS, Sigma, Europe), and nonspecific binding sites were blocked with PBS containing bovine serum albumin (BSA, Sigma, Europe). Wells were washed again, and added with biotinylated trout microsomes (except for the blank wells). Samples or standards (with adjusted protein concentration) were then added to the wells. Competition was allowed to take place and after five washing steps, extravidin-HRP (Sigma, Europe) was added to all the wells. The plate was then incubated and the wells were washed again using PBS. Chromogen TMB (Pierce, United Kingdom) was added to all the wells and the plate was incubated in obscurity. Sulfuric acid (Sigma, Europe) was added to the wells to stop the reaction. Optical density was measured in the 96-well plates at 450 nm using a Packard Spectracount microplate reader.

1.4.2. Laboratory studies

The starfish were measured as described for the field study. Except where otherwise stated, the starfish were fed with mussels of suitable size according to the relation established by Sommer *et al.* (1999) between the size of the starfish and the average size of eaten mussels (mussels of 2 to 50 mm shell length for starfish of 5 to 65 mm

ray length). All experiments were performed on juvenile starfish (5 to 35 mm ray length at the beginning of the experiments), i.e. individuals that are below the size of maturity in these populations (Joly et al. 2003).

1.4.2.1. Influence of food supply on juvenile growth

At Aber, despite the presence of an abundant mussel bed, most starfish, and particularly juveniles, are found in an accumulation of pebbles where prey abundance seemed to be low. Therefore, juveniles (collected in Feb. 01 at Aber, 9.8 ± 3.6 mm ray, mean ± standard deviation) were reared during 5 months and fed on three different diets: (1) mussels *ad libitum*, (2) mussels at a level corresponding of half of satiety and (3) "Aber food supply" obtained by scrapping an area of 0.28 m² of pebbles from Aber. Starfish fed *ad libitum* were provided daily with an excess of mussels, the number of eaten mussel being regularly counted all through the experiment to adjust to half satiety food supply (providing half the number of eaten mussel in the *ad libitum* treatment). "Aber food supply" was renewed before complete prey disappearance. Juveniles were placed in an open system in six 30L aerated aquaria (12 starfish per aquarium, 2 replicates for each diet). Starfish size was measured monthly. Salinity stayed at 34-35 PSU and the temperature rose from 10°C in February to 16°C in late June. In half satiety fed starfish, high mortality levels were recorded starting in the second month; at the end of the experiment 54% of the starfish had died while 20% of starfish fed with "Aber food" and 4% of *ad libitum* fed starfish had died. In the half satiety treatment, a few casualties were due to cannibalism.

1.4.2.2. Emersion influence on juvenile growth

To test the influence of tidal rhythm on juvenile starfish growth, juveniles (collected in Oct. 02 at Knokke, 23.2 ± 4.3 mm ray, mean ± standard deviation) were reared during about 3 months (from Nov. 18th, 2002 to Feb. 28th, 2003) in 2 closed systems. One was designed to allow emersion of the starfish (starfish were emersed in 15 minutes and immerged in 4 minutes); the emersion periods were adjusted to the predicted periods when the natural Knokke population was uncovered (i.e. each time the sea level was lower than 1.70 meters in Knokke). In the second system, the starfish were kept immerged.

In each system natural sea water (200 liters) circulated continuously. Each system was composed of 3 aerated aquaria (20 starfish per aquarium), a filtering unit (gravel and activated charcoal), a stock water tank and a pump. In the emersion-immersion system the pump was switched on or off by a timer. Seawater temperature was adjusted to the field temperature: 10-12°C during the first 3 weeks, then 6°C until the end of the experiment. Salinity, pH and NO_3^- concentrations were measured every

day. Salinity was adjusted to 34-35 PSU. When NO_3^- concentration levels rose above 0.3 mg/l the seawater content of both systems was renewed.

In both systems, the starfish were fed *ad libitum* with mussels and the number of eaten mussels was recorded every week. The size of the starfish was measured monthly. At the end of the experiment, the righting time of the starfish was measured as the time in seconds required by a starfish to turn back after being placed on its aboral face (righting time is a commonly used indicator of stress and well-being of asteroids, Lawrence & Cowell 1996). During the 5th and 6th week of the experiment, 1 starfish died in emersion condition and 3 starfish presenting body wall necrosis were removed from the immersion condition. No further mortality took place.

1.4.2.3. Influence of salinity variations on juvenile growth

In Knokke and Breskens, populations are submitted to daily varying salinities due to the influence of the Scheldt river. To test the effect of such salinity variations, small starfish (collected in Sept. 03 at Aber, 24.5 ± 2.3 mm ray, mean ± standard deviation) were maintained in 5 experimental systems during 15 days (Table 2). The open system (control) was continuously supplied with water pumped in the Bay of Brest (France). The 4 closed systems (100 l) had a continuous flow of recycled natural seawater and a filtering unit of activated charcoal. At each salinity change in VS30 and VS26 systems (systems where starfish were exposed to varying salinity, see Table 2), the water content of all closed systems was renewed. Each system was aerated. Temperature of the systems was maintained at that of the open system, i.e. 18°C.

At the beginning of the experiment, the mean ray length of each starfish was measured (mean of the 5 rays measured in mm) and the starfish were weighed (emersed wet weight). In each system, 20 starfish of similar ranges of size and weight were placed in a 30 l aquarium, where each starfish was individualised in a small separated tank (0.5 l) with drilled holes to ensure water circulation. They were each provided with 10 mussels. At every seawater change, the eaten mussels were counted and replaced by mussels of the same size. At the end of the experiment, the mean ray length and the weight of each starfish were measured. Their righting time was measured at the last salinity they were submitted to (i.e. 34 PSU except for CS26 for which it was 26 PSU). There was no mortality.

1.4.2.4. Influence of sea water temperature on juvenile growth

To test the influence of seawater on juvenile starfish growth, juvenile (collected in Oct. 03 at Knokke, 25.2 ± 4.1 mm ray, mean ± standard deviation) were reared during 5 months (Oct. 03 to March 04) at 3 temperatures, 8-9°C, 13°C and 16-17°C, in 3 closed systems (1500 l, continuous recycled water flow). In each system 60 starfish were placed in three 30 l aerated aquaria (20 starfish per aquarium). They

were fed *ad libitum* with mussels. The salinity of each system was kept at 32 PSU. Water was filtered by a gravel and activated charcoal filtering unit. Salinity, pH and NO_3^- concentration levels were controlled and adjusted by renewing seawater with natural seawater or by adding tap water. Starfish size was measured monthly.

In January (mid-experiment), half of the starfish were removed for an other analysis unlinked to the present study; care was taken not to modify the size distribution in each aquarium.

1.4.3. Data analyses

Available food in the different locations was compared using a two-way ANOVA. Biomasses were log-transformed ($X' = \log(X+1)$) to ensure homoscedasticity (Zar 1996). Heavy metal concentrations in the body wall and in the pyloric caeca were compared using a one-way ANOVA (factor location). In experimental and field studies, growth of juveniles was modeled by linear regression of the cohort mean size against time. No other relationship was used due to the small number of data points. Slopes of the linear regression, i.e. growth rates of juveniles, were compared with ANCOVA (Sokal & Rohlf 1995).

In experimental studies where several aquaria were used for each condition (emersion and temperature influences), differences in size or feeding rates were tested using a nested ANOVA with "experimental condition" and "aquaria nested within experimental condition" as factors. The nested factor allows to distinguish possible discrepancies between pseudo-replicates within each experimental conditions (Zar 1996).

Differences between measured DLC concentrations or CYP1A IPP induction in the different stations were tested using the non-parametric Kruskal-Wallis test followed by a multiple comparison test of the means (Dunn test, Conover 1980). Regressions between the concentrations measured in sea star pyloric caeca and mean induction of CYP1A IPP measured in corresponding individuals were tested using simple linear regression procedures (Zar 1996), applying a Bonferroni criterion to the significance level. The level of significance for statistical analyses was always set at a = 0.05.

1.5 RNA/DNA ratio in adults

For RNA/DNA analysis around 25 individuals of *A. rubens* and *E. acutus* were collected in the same Sørfjord stations as for gamete quality assessment (Table 1). All individuals were processed within 5 h after collection. Prior to dissection, arm length of the starfish, from arm tip to mouth, and ambital diameter of sea urchins were measured; all individuals were weighted. Size and weight of starfish used in the present study ranged between 54 to 100 mm and 20 to 132 g fresh weight (FW), respectively; those of sea urchins between 42 to 100 mm and 40 to 450 g FW.

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 -80ºC 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 specimens as for RNA/DNA analysis, oven dried at 60ºC for 2 d and stored for metal analysis. Starfish were in the pre-spawning stage (i.e. ripe and ready to spawn).

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 Belchier et al. (2004) protocols.

Prior to analysis, deep-frozen samples (-80°C) were freeze-dried for 48 h. Samples were then grinded using a mortar and a pestle, to provide an homogeneous powder. Around 2 to 3 mg DW of pyloric caeca and gonad tissues (from *A. rubens* and *E. acutus*, respectively) were analysed, as well as around 20 mg DW of body wall (from both species). Samples were extracted using Tris-EDTA buffered (5 mM Tris-HCl, 0.5 mM EDTA, pH 7.5) 0.01% Sodium dodecyl sulphate (SDS SIGMA L4390, Saint Louis, MO, USA). They were homogenized on ice and then centrifuged (3500 g, 15 min, 4ºC). From each sample, four aliquots of supernatant were transferred to four wells on a 96-multi-well plate: two replicates were used to determine total nucleic acid concentration and two for DNA determination, after RNA digestion by RNase (Sigma R-6513; 30 min at 37ºC and 30 min to cool down to room temperature). There was an addition of EB 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). Fluorescence was read using Spectrofluor Plus microplate reader from Tecan© (Mannedor/Zurich, Switzerland) Excitation and emission wavelengths were 365 nm and 590 nm, respectively. The RNA fluorescence was calculated by subtracting DNA fluorescence reading from the total nucleic acids 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 μg RNA mg^{-1} sample DW divided by μg DNA mg^{-1} sample DW.

2. Bivalves

Between 1996 and 2002, samples of wild mussels of 3-4 cm shell length were routinely collected from 4 sites in the Western Scheldt estuary (Figure 1). The selected sites following both pollution and salinity gradients. The salinity at the sites ranged from 18 ppt at Hansweert at the upper limit of mussel distribution to full seawater with 34 ppt at Westkapelle near to the mouth of the estuary (Figure 1). Samples for annual variation were collected during the summer months between July-September, in order to sample the mussels in the post-spawning period and also in order to make comparisons with JMP data.

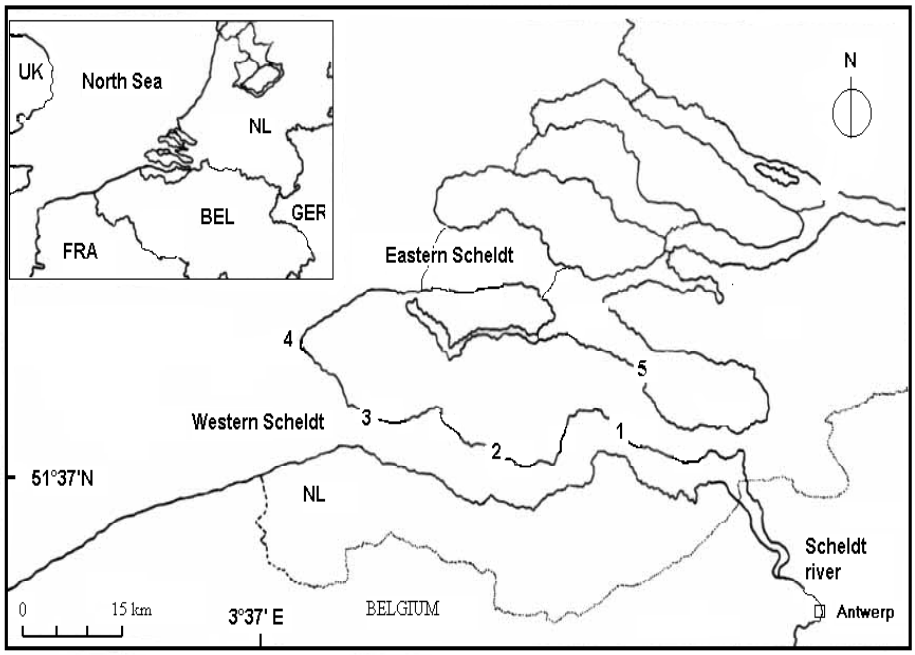


Figure 1. Map of Western Scheldt estuary and the Eastern Scheldt tidal basin (The Netherlands) showing the five sampling sites; 1 – Hansweert, 2 – Ellewoutsdijk, 3 – Vlissingen, 4 – Westkapelle and 5 – Wemeldinge

For seasonal variations, two sites were included in the study; Hansweert the most polluted site in the WS and Wemeldinge in the relatively less polluted Eastern Scheldt. The two sites were selected in order to investigate whether seasonal variations were depended on the level of metal pollution in the environment. Samples were taken in October and December of 1999 and in February, April and August 2000.

During sampling, 25-35 mussels were always collected from each site close to the water line during low tide. Samples were then transported on ice in a cool box to the laboratory. Each individual was cleaned and then dissected with a clean scalpel blade to separate the soft tissues from the shells. Soft tissues of each individual were then placed in a polypropylene vial. Samples were dried in the oven for 72 h at 60 °C, after which tissue dry weight was determined. 5 ml of ultra pure concentrated (69%) HNO_3 and 0.25 ml 27% H_2O_2 were added. Samples were digested by heating in a microwave oven during four consecutive steps of 5 minutes at 80, 160, 240 and 320 watts. After digestion, samples were diluted by adding 30 ml Milli-Q water in each vial. Metal concentrations in the final solutions were determined by ICP-AES (Cu, Fe, Mn, Zn) and ICP-MS (Cd, Co, Cr, Ni, Pb). Yttrium was used as an internal standard to correct for interference from high dissolved solids arising from high salt content and tissue matrix effects. Samples of reference material of mussel tissue (CRM 278R, Institute of Reference Materials and Measurements, European Commission, Geel, Belgium) were always included with each batch of samples for verification of the measurements. Recoveries in the reference materials were checked against certified values and measurements were only accepted if the recoveries in the reference samples were within 10% of certified values (Table 1).
For statistical analysis, annual tissue metal concentrations in mussels were analysed using a 2-way ANOVA with *year* and *site* as independent factors. 2-way ANOVA was also applied to test the effects of *season* and *site* on metal concentration in mussels. In the latter case, only two sites (Hansweert and Wemeldinge) were used in the analysis.

3. Sediment-Associated Microbial Communities (SAMC)

The aim of this part of the work is to understand the degree to which metal contaminants influence SAMC in the marine environment. A good approach to the problem is the study of extremely contaminated places ("hot spots" of pollution) such as the Sørfjord in Norway. The Sørfjord is about 38 km long, 1-3 km wide and maximum 390 m deep (Fig.5). It is an exceptional hot spot of heavy metal contamination because large smelters located at the head of the fjord have discharged huge quantities of Zn, Cd and Pb into the surrounding environment for more than 65 years. Figures for 1980 indicated that 1387 tons of Zn, 329 tons of Pb, and 14.6 tons of Cd were discharged into the fjord during this year. Although inputs were significantly reduced in 1986, heavy metals in sediments still show a clear gradient from very high concentrations in the inner fjord (station S1) to background levels at the opening of the fjord on the sea (station S4) (Fig. 5) (Coteur et al. 2003b, Temara et al.1998). The aim of the work presented here was to study the SAMC along the Sørfjord.

3.1. Study site and sampling

The sampling stations (S1-S4) were located as shown in Fig. 1. Sampling was done in April 2003. S1 was situated 100 m from a zinc smelter and is the most contaminated site (Temara et al. 1998, Coteur et al. 2003). Mean concentrations of metals observed in S1 in the total sediments (DW) were 60 μg/g for Cd, 100 μg/g for Cu, 800 μg/g for Pb, and 100 μg/g for Zn (Coteur et al. 2003). S2 was located in front of a titanium smelter, with S3 12 km further north and S4 in the neighbouring fjord in the direction of the open sea. S4 is considered as a control site. Sediments were collected in March 2003 by SCUBA divers at a depth of 15 m. The salinity of the seawater just above the sediments was 31‰ and its temperature was 5°C. The top 3 cm of the sediments were collected using acid-washed 500 ml plastic vials. Six replicate samples were collected at each site.

3.2. Sediment and microbial analyses

Bioavailable metals were determined as described in Gillan (2004) and a contamination index (CI) was determined for each site according to Feris et al. (2003). Organic carbon was determined by carbonisation at 450°C for 4h. For direct counts sediment samples (n=4) were fixed with paraformaldehyde, treated by mild sonication (Gillan 2004), placed on filters, and counted as described by Pernthaler et al. (2001). The mean number of bacteria counted per filter was 570 ± 66. Cd resistance was evaluated by the plate count method, using Nutrient agar plates enriched with $CdCl_2$. For cloning and sequencing of the 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE), and phylogenetic analyses, the method described in Gillan et al. 1998 was used. Fluorescent in situ hybridization (FISH) with domain-specific probes was done on triplicate samples according to Pernthaler et al. 2001. The number of DAPI-stained cells that were counted for each probe at each site was 1385 ± 388 cells.

RESULTS AND DISCUSSION

1. Echinoderms

1.1. Gamete quality

1.1.1. Metals in the gonads of starfish

Cadmium, lead, zinc, and copper concentrations in the gonads of starfish sampled in four sites along the Sørfjord are given in Table 6. Concentrations of Pb, Zn, and Cu did not differ significantly between starfish from the different sites. Zn and Cu were significantly more concentrated in, respectively, female and male gonads ($p_{ANOVA} \leq 0.03$). Lead concentrations in female gonads from S1 starfish were significantly higher than those of male gonads in the same site and female gonads from SN3 starfish ($p_{Tukey} \leq 0.024$). All effects (site, sex, interaction) significantly affected Cd concentrations ($p_{ANOVA} \leq 10^{-3}$). Mean multiple comparisons (Tukey test) showed that this is only due to the high Cd concentration in male gonads in S1 which significantly differed from Cd concentrations measured in the gonads from starfish of all other sites and from female gonads from S1 starfish. (No other mean comparisons were significant.)

Metal concentrations in the pyloric caeca and the body wall did not differ between sexes but differed according to the site in which the starfish were collected (except Cu), as previously reported (Coteur et al. 2003b). Cd, Pb, and Zn concentrations were always significantly higher in one or both of these compartments in the starfish from S1 (data not shown).

Table 6. Metal concentrations (µg/g DW; mean ± sd) in gonads of *Asterias rubens* from the Sørfjord according to the site and sex (f: female, m: male)

Site-Sex	Cd	Pb	Zn	Cu
S1-f (n=4)	0.222 ± 0.059	20.70 ± 15.18	217.3 ± 100.3	4.54 ± 0.51
S1-m (n= 6)	1.606 ± 0.662	2.70 ± 1.74	81.1 ± 6.4	5.76 ± 0.68
S2-f (n= 5)	0.187 ± 0.099	5.77 ± 3.45	249.3 ± 118.7	4.76 ± 2.34
S2-m (n= 4)	0.123 ± 0.031	14.86 ± 13.14	97.1 ± 22.5	4.95 ± 0.37
SN3-f (n= 6)	0.167 ± 0.042	2.35 ± 0.84	301.4 ± 59.1	4.25 ± 0.21
SN3-m (n= 3)	0.121 ± 0.024	1.62 ± 0.51	67.6 ± 6.0	4.86 ± 0.40
S4-f (n= 6)	0.125 ± 0.032	7.17 ± 7.30	309.5 ± 27.5	4.69 ± 1.03
S4-m (n= 2)	0.735	12.61	50.8	6.14

1.1.2. Egg size and morphology; fertilization success

Egg size (area) did not differ significantly according to the site where the starfish were collected ($p_{Nested\ ANOVA} > 0.25$, n= 8073; grand mean ± sd= 8060 ± 907 µm^2) nor did egg morphology (ratio between the largest and smallest diameter) ($p_{Nested\ ANOVA} > 0.25$, n= 8073; grand mean ± sd= 0.881 ± 0.057).

The germinal vesicle was never observed except in obviously damaged eggs (<1% of eggs in all sites). Fertilization success was higher than 95% in all sites.

1.1.3. Sperm motility

The dry sperm concentrations of *A. rubens* varied between 1.04 to 9.75x10^{11} sperm cells mL^{-1}. No differences between station were found (p=0.70). The velocity parameters varied between 193.9 and 31.4 µm s^{-1}, for VCL, 97.5 and 26.4 µm s^{-1}, for VSL, and 182.1 and 27.5 µm s^{-1}, for VAP. No significant differences were found between stations (p≥0.244).

1.1.4. Discussion

Mature *Asterias rubens* were easily collected all along the heavily contaminated Sørfjord. Gonads of these starfish appear contaminated by Cd and Pb when compared with those from the Southern Bight of the North Sea. Cd concentration is higher in male gonads of S1 while Pb concentrations are elevated in both sexes all along the fjord. In contrast, Zn and Cu have similar levels in the Sørfjord and in the Southern North Sea. However, despite the occurrence of significant gradients of metal contamination in the sediments from the head of the fjord towards its opening, which are reflected in some organs of the starfish (pyloric caeca, body wall) (Coteur et al. 2003b), metal concentrations in the gonads do not differ in starfish collected in the different sites (except for Cd in S1 males). This lack of variation indicates that metal translocation to female gonads is probably tightly controlled. It very probably accounts for the similarities in egg morphology and fertilization success between starfish from the different sites.

The present results contrast with those reported by den Besten et al. (1989). In this study, after 5 months (encompassing the whole gametogenesis period) of mesocosm exposure to 25 µg Cd/l, gonads of male and female starfish contained respectively 0.15 ± 0.06 and 0.70 ± 0.09 µg Cd/g DW, to be compared with 1.61 ± 0.66 µg Cd/g DW in S1 males and 0.22 ± 0.06 µg Cd/g DW in S1 females. The mesocosm contaminated females produced a significantly lower proportion of oocytes that had completed germinal vesicle breakdown at the moment of spawning (den Besten et al. 1989). This was not the case in the present study, emphasizing the differences between aquarium or mesocosm experiments and field situation.

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, were observed. As metal analysis in the same gonads was impossible due to spawning induction, it can be argued that depuration occurred while the starfish were kept in uncontaminated water (no more than two weeks). However, evidence from the literature show that, for instance, starfish gonads do not eliminate Pb and Co over such short periods of time (Temara et al 1998, Warnau et al 1999). On the contrary, gonads appeared as receptacle for metals. Furthermore, spermatogenesis in starfish is a cyclic process. During this process, spermetogonia are stored in the gonad lumen until spawning. Therefore, the analysed sperm was anyway submitted to contaminated metals. Thus, the present results indicate that sperm motility is not impaired in heavy field contaminated starfish. These results suggest that *A. rubens* is able to reproduce in environments heavily contaminated by metals.

1.2. Larval survival

A single factor ANOVA was performed on the best results obtained for each population, each year (Table 7). In 2002, larval viability in the population from Scharendijke was similar to that in the Brittany populations except that the number of dead larvae was lower in Brittany. In 2003, percent of dead and viable larvae discriminated the North Sea and Brittany populations. The North Sea populations produced more dead larvae and less viable larvae. Differences in the proportion of normal larvae were more subtle; it was higher in Piriac than in Ambleteuse, the other populations (Aber, Knokke and Scharendijke) showing intermediate results.

Table 7: Geographical variations in larval viability. Results of the single factor ANOVA on the best results obtained for each year in each population and subsequent Tuckey multiple mean comparison test. As the data sets were used for other comparisons a Bonferroni criterion was applied and α = 0.017. Significant probabilities are presented in italics. Percentages of larval type produced by genitors of locations (Aber, AB, Ambleteuse, AM, Knokke, KN, and Scharendijke, SC) that are underlined do not significantly differ.

Year	Studied population (best month), n = total number of fertilization Results of the statistical analysis		
2002	PI (May), AB (May) and SC (April), n = 36		
	% D	$p < 10^{-9}$	SC > <u>PI AB</u>
	% N	p = 0.036	<u>PI AB SC</u>
	% V	p = 0.12	<u>SC PI AB</u>
2003	PI (April), AB (May), AM (April), KN (April) and SC (April), n = 60		
	% D	$p < 10^{-9}$	<u>KN SC AM</u> > <u>PI AB</u>
	% N	$p = 1.4\ 10^{-5}$	<u>PI AB KN</u> <u>SC AM</u>
	% V	$p = 1.1\ 10^{-7}$	<u>AB PI</u> > <u>SC KN AM</u>

Heavy metal concentrations were determined in the pyloric caeca and gonads. Results of the principal component factor analysis performed with heavy metal concentrations in each body compartment as variables and subsequent ANOVA are shown in Table 8; 64.6% of the total variance was explained by 3 factors which grouped all variables. ANOVA on factor scores showed significant differences between locations for each factor except for factor 3 (lead in both organs and copper in the gonads). Ambleteuse starfish appeared as the less contaminated. They presented lower levels of copper and zinc in their pyloric caeca than starfish of all other populations. Their gonads were less contaminated by cadmium and zinc and

their pyloric caeca by cadmium**.** Cadmium, copper and zinc concentrations in the gonads of *A. rubens* were shown to be related to sex (Dubois et al. 2004, Temara et al. 1997). As the factors issued from the principal component factor analysis grouped heavy metals contamination in the gonads as well as in the pyloric caeca, differences related to sex might have been hidden. Therefore ANOVA were performed on the heavy metal gonadic concentrations; factor "sex" was never significant ($p \leq 0.017$) reinforcing the results of the principal component factor analysis.

Table 8: Contamination levels. Results of the principal component factor analysis performed with heavy metal concentrations in each body compartment as variables (n = 8; heavy metals: cadmium, Cd, copper, Cu, lead, Pb, and zinc, Zn; body compartments: pyloric caeca, PC, gonad, G.). Results from the two factor ANOVA and subsequent Tuckey multiple mean comparison test on the factors loadings (α = 0.017). Significant probabilities are presented in italic. Factors loading from locations (Aber, AB, Ambleteuse, AM, Knokke, KN, Piriac, PI, and Scharendijke, SC) that are underlined together do not significantly differ.

Factor	Percent of total variance explained	Groups of variables (Sign of the relationship)		p ANOVA	Ranking of locations (decreasing values)
F1	26.4	Cd in G and PC (+) Zn in G (+)	Location Sex Interaction	*20 10^{-3}* 0.30 0.13	KN PI AB SC AM
F2	17.9	Cu and Zn in PC (+)	Location Sex Interaction	*3 10^{-5}* 0.50 0.55	PI SC KN AB > AM
F3	20.3	Pb in G and PC (+) Cu in G (+)	Location Sex Interaction	0.050 0.86 0.09	
total	64.6				

The relationship between heavy metal contamination (mean for each body compartment) and larval viability in 2003 (mean percentage of dead and viable larvae) was studied by a principal component factor analysis with varimax rotation (Table 9); 95.6% of the total variance was explained by 3 factors which grouped all variables. Larval viability was positively related to copper concentrations in both organs and to a lesser extends to zinc concentration in the gonads. This was mainly due to the elevated concentration of these metals in the considered body compartments of Brittany starfish.

Table 9: Relationships between heavy metal contamination and larval viability in 2003. Results of the principal component factor analysis performed with mean heavy metal concentrations and mean percentages of dead (%D) and viable (%V) larvae as variables (n = 10; heavy metals: cadmium, Cd, copper, Cu, lead, Pb, and zinc, Zn; body compartments: pyloric caeca, PC, gonad, G.)

Factor	Percent of total variance explained	Groups of variables (Sign of the relationship)
F1	37.1	Cd in G and PC (+) Zn in G and PC (+)
F2	36.8	%V (+) and %D (-) Cu in G and PC (+) Zn in G (+)
F3	21.7	Pb in G and PC (+) Cu in G (+)
total	95.6	

The viability of *Asterias rubens* larvae varied with month, year and population. According to the maturity periods determined for these populations, fertilization assays were conducted during 2 months: March and April in the North Sea, April and May in Brittany. In the North Sea, larval viability was better at the end of the study period, indicating an insufficient ovocyte maturity in March. In Brittany, the best offspring was obtained on different months according to the year. This reflected the fluctuation of the spawning period between geographical areas and years (Joly et al unpublished, Nichols & Barker 1984a).

Interestingly, larval quality might seriously differ between years in the same population. This was particularly true for Scharendijke offspring. It is noteworthy that the larvae were still in the endotrophic stage when they were analysed. So, larval food supply was not involved in this phenomenon. This poor larval production in 2003 was general in the 3 studied North Sea populations. This ruled out a local effect like pollution. This is further supported by the fact that larvae from Ambleteuse genitors did not score better despite their lower contamination both by metals (this study) and PCBs (Danis et al. 2006). Interestingly, in Knokke, where a long-term survey of population characteristics is carried out, the lowest gonad index in the period 2000-2003 was recorded that year (Pernet et al, unpublished). This suggested that genitors condition could influence larval quality. However, the effect of a waterborne toxic component could not be ruled out, due to the necessary use of sea water from different field origin in the larval assays.

Offspring quality appeared to be positively related to the level of copper in the pyloric caeca and zinc and copper in the gonads of the genitors. This could be due to the high concentration of these metals in the organs of Brittany starfish. Both are essential metals (Harrison & Hoare 1980, Kendrick et al. 1990) and their pyloric and gonadic loads are believed to be controlled. In sea urchin eggs, cytochrome oxydase is involved in respiratory activation at fertilization (Giudice 1986) and copper is a

cofactor of this enzyme (Harrison & Hoare 1980). An increase in egg copper load might be beneficial to the respiration of embryos. However, the relationship between offspring quality and these 2 metal levels in the gonad could only be a side-effect of the poor offspring quality in North Sea population in 2003. Offspring quality in Scharendijke in 2002 was as good as in Brittany populations despite similar copper and zinc levels in this site over several years (Danis et al. 2006, Temara et al. 1997).

1.3. Embryo assays

1.3.1. Toxicity of Polycyclic Aromatic Hydrocarbons

The frequencies of larval morphotypes in benzo-α-pyrene-exposed embryos are reported in Table 10 for *P. miliaris* and Table 11 for *A. rubens* as examples. Complete results are available in the "Belgian Marine Data Centre" (http://www.mumm.ac.be/datacentre/).

PAH and UV-B were not toxic by themselves. For both echinoderms, exposition to PAH in darkness did not induce larval mortality. However, the PAH toxicity became obvious when they were combined with UV-B radiation. The photo-toxicity of some PAH was already observed using larvae of mollusk (*Mulinia lateralis*, Pelletier *et al.* 1997; *Crassosteras gigas*, Lyons *et al.* 2002) and early embryonic stages of echinoid (*Lytechinus pictus*, Steevens *et al.* 1999).

The combination of PAH and UV-B induced three profiles of dose-dependant response: neutral, sub-lethal and lethal. The "neutral" profile is characterized by the lack of obvious toxicity; the rates of normal and viable larvae for all tested concentrations were usually identical to controls (Dunnett test, α= 0.05). The "lethal" profile showed massive larval mortality but only at the higher tested concentrations. The "sub-lethal" profile could also present larval mortality at high concentrations but is mainly characterized by a drastic reduction of the rate of normal and viable larvae at intermediate concentrations.

Tests performed using *P. miliaris*, which constitutes our principal model, induced these three profiles of larval response. In function of this response, it was possible to classify the PAHs by growing order of toxicity corresponding (from left to right):

(NA, PH) < ANT < BaP < (BaA, Py, FLAN)

Table 10. *Psammechinus miliaris*: Toxicity of benzo[α]pyrene in darkness or under UV-B radiation. Percentages (mean ± s.d.) of each morphotype after exposition to single PAH throughout embryogenesis (72 h). 6 replicates by exposure, 100 larvae scored by replicate. **SWC**: control in filtered sea water; (**+**): all larvae died during the experiment; **nd**: no data.

		Psammechinus miliaris					**Benzo[*a*]pyrene**			
		Experiments performed in darkness					**Experiments performed under UV-B radiation**			
Conc. (µg/l)		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Delayed**	**Highly Delayed**	**Ab-normal**		**Normal**	**Delayed**	**Highly Delayed**	**Ab-normal**
0.01	m.	18.17	48.00	5.00	28.83		29.00	43.33	1.33	26.33
	s.d.	7.86	9.12	4.38	3.60		9.19	9.91	2.34	6.38
0.1	m.	9.83	58.00	7.67	24.50		22.67	43.00	1.50	32.83
	s.d.	5.15	6.84	3.98	4.76		3.78	1.90	1.52	2.71
0.5	m.	11.83	55.67	3.83	28.67		15.83	44.50	1.50	38.17
	s.d.	2.93	6.19	1.33	4.50		1.72	2.59	1.05	2.71
1	m.	19.20	48.20	1.60	31.00		30.67	36.00	2.00	31.33
	s.d.	5.97	6.10	1.52	5.43		3.98	2.10	3.16	3.08
5	m.	14.33	48.50	6.00	31.17		2.20	33.40	19.80	44.60
	s.d.	4.68	5.36	2.90	5.88		3.90	15.32	3.70	17.95
10	m.	16.00	54.83	6.33	22.83		+	+	+	+
	s.d.	4.73	5.64	3.93	5.19		+	+	+	+
100	m.	23.33	40.50	3.83	32.33		+	+	+	+
	s.d.	8.43	5.50	1.72	6.02		+	+	+	+
	m.	22.33	44.33	3.67	29.67		21.83	44.83	8.50	24.83
SWC	s.d.	2.50	5.09	1.37	4.23		4.26	4.45	4.14	5.12

Naphtelene (NA) and phenantrene (PH), characterized by a "neutral" profile, are at the bottom of this scale. Both are LPAHs (*i.e.* Low molecular weight PAH) including only two (NA) or three (PH) benzene nuclei. Indeed, LPAHs are not very toxic for the environment (Napgal 1993). The other extremity of the scale is formed by three HPAHs (*i.e.* High molecular weight PAH) which are considered as more toxic than the LPAHs (Napgal 1993). These three HPAHs, including four benzene nuclei, induced massive larval mortality at concentrations equal and superior to 5 µg/l. Following our own classification, PAHs exhibiting a "sub-lethal" profile are intermediary. This kind of profile was induced by two PAHs: anthracene (ANT), a LPAH including three benzene nuclei, and benzo[α]pyrene (BaP), a HPAH including five benzene nuclei. Exposition to 5 µg of ANT or BaP per liter reduced significantly the rate of normal and viable larvae (*i.e.* sub-lethal effect). Higher concentrations could induce larval mortalities. However, these mortalities were induced by lower concentrations of BaP than ANT. For this reason, ANT was placed near the "neutral" PAHs and BaP near the "lethal" PAHs.

Table 11. *Asterias rubens*: Toxicity of benzo[α]pyrene in darkness or under UV-B radiation. Percentages (mean ± s.d.) of each morphotype after exposition to single PAH throughout embryogenesis (72 h). 6 replicates by exposure, 100 larvae scored by replicate. **SWC**: control in filtered sea water; (**+**): all larvae died during the experiment.

		Asterias rubens					**Benzo[*a*]pyrene**			
		Experiments performed in darkness					**Experiments performed under UV-B radiation**			
Conc. (µg/l)		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Delayed**	**Highly Delayed**	**Ab-normal**		**Normal**	**Delayed**	**Highly Delayed**	**Ab-normal**
0.01	m.	25.50	42.50	4.17	27.83		43.17	28.33	5.50	23.00
	s.d.	8.64	3.73	1.60	6.49		7.03	3.50	2.26	4.98
0.1	m.	34.00	35.67	3.00	27.33		38.00	30.50	7.00	24.50
	s.d.	5.48	4.37	0.63	4.84		10.22	3.94	2.68	6.57
0.5	m.	35.83	36.50	6.00	21.67		36.17	39.67	3.17	21.00
	s.d.	7.76	6.06	1.10	7.53		4.92	6.50	1.47	3.90
1	m.	43.17	33.83	2.83	20.17		44.83	31.50	2.17	21.50
	s.d.	5.71	2.40	2.32	4.62		7.55	4.76	0.98	4.59
5	m.	36.83	38.00	2.83	22.33		29.67	21.67	12.67	36.00
	s.d.	6.01	2.53	2.14	5.50		15.04	6.59	7.69	12.00
10	m.	39.17	32.67	4.33	23.83		0.00	6.80	38.20	55.00
	s.d.	5.19	2.42	2.80	3.19		0.00	3.70	10.06	11.31
100	m.	40.50	36.50	2.50	20.33		+	+	+	+
	s.d.	5.61	5.54	2.07	4.76		+	+	+	+
	m.	46.00	30.83	4.83	18.33		52.83	22.83	3.00	21.33
SWC	s.d.	8.72	8.13	3.13	2.80		3.19	3.13	1.10	4.89

PH and ANT are characterized by the same generic formula and have the same molecular weight. However, PH is associated to the "neutral" PAHs and ANT to the "sub-lethal" ones. It seems so that the conformation of the molecule (*i.e.* straight for ANT *versus* in arc of circle for PH; see Table 4) directly influence its toxicity.

It is surprising to note that BaP, considered as one of most dangerous PAH for the living beings (OSPAR 2000), is not associated to the group of "lethal" PAHs. Steevens *et al.* (1999) already reported similar observations using early embryonic stages of the echinoid *Lytechinus variegatus*. These authors observed a reduction of the rate of development of the 4 cells stage from the concentration of 1 µg/l and a complete arrest of development at 50 µg/l. These effective concentrations are similar to the ones obtained during our study with older larval stages.

Tests performed using *A. rubens* larvae appeared to be less sensitive. Only two types of dose-dependant profiles were observed: neutral and sub-lethal. Following

the same conventions, the four studied PAHs can be classified by growing order of toxicity (from left to right):

ANT < (PY, FLAN, BaP)

With *A. rubens* larvae, the difference between LPAHs and HPAHs became more obvious. There were two main differences with the results obtained with *P. miliaris* larvae. The LPAH ANT did not induce a "sub-lethal" profile but a "neutral" one. However, significant reductions of the rates of normal and viable larvae were observed at the highest tested concentrations (*i.e.* 100 µg/l). With a larger range of concentration, the resulting profile could have been different. The sub-lethal profiles of HPAHs PY, FLAN, and BaP are also characterized by massive larval mortalities. However, those mortalities are induced at concentrations a hundred times higher than for *P. miliaris* larvae.

The comparison between the induced larval morphotypes allowed to compare the impact of PAHs with "sub-lethal" profiles. PAHs did not induced specific abnormal morphotypes but altered the relative proportions of existing morphotypes at some concentrations, just under the lethal ones. For *P. miliaris*, ANT and BaP increased the rates of highly delayed larvae and, to a lesser extent, the ones of abnormal larvae. For *A. rubens*, highest tested concentration of ANT (*i.e.* 100 µg/l) increased the rate of abnormal larvae. By opposition, exposition of *A. rubens* larvae to high concentrations of PY, FLAN or BaP increased the rates of highly delayed and abnormal larvae (until 80% of abnormal larvae in response to 10 µg/l of PY). At sub-lethal concentrations, our results indicated that PAHs mostly induces delay of development in *P. miliaris* and abnormalities in *A. rubens*. This difference of sensibility between the two echinoderms could result from specific mechanisms of protection against contaminants. Protection mechanisms against PAHs exist at the cellular level in echinoderms (*e.g.* cytochrome P450, Snyder 2000). Others mechanisms are possible like MAAS systems (mycosporine-like amino acids systems, Epel *et al.* 1999) or anti-oxidant agents (Lesser & Barry 2003).

1.3.3. Toxicity of the *Phaeocystis* colonies and derived products

1.3.3.1. Pre-test: Methanol toxicity.

Methanol was used as solvent carrier to transfer the extracted organic matters into the FSW. Concentrations of methanol in FSW from 0.02% to 1% do no effect the further development of *A. rubens* larvae (Dunnett test, $\alpha = 0.05$).

1.3.3.2. Toxicity of Belgica's sea water.

A reduction of the rates of normal or viable larvae was observed during some experiments, v*iz.* when the higher concentration of FBSW or of organic extracts was tested. This concentration actually corresponds to the original concentration in sea

water at the moment of the sampling. The observed allelopathic effect decreased rather regularly with increasing dilution.

Table 12. *Psammechinus miliaris*: Percentages (mean ± s.d.) of each larval morphotype after exposition to filtered waters from station VB8 throughout embryogenesis (72 h). 6 replicates by exposure, 100 larvae scored by replicate. **FSW**: control in filtered sea water. There were significant differences between the results in bold and the corresponding control (Test performed on normal and viable larvae only; Dunnett, α= 0.05).

		Inverted filtration (Intact colonies)					**Normal filtration (Broken colonies)**			
		Filtered water					**Filtered water**			
Dilution		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Abnormal**	**Delayed**	**Viable**		**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**42.00**	38.00	20.00	**80.00**		**67.67**	16.50	15.83	**84.17**
	s.d.	**10.33**	6.23	5.90	**5.90**		**5.72**	2.95	3.87	**3.87**
C/2	m.	**58.17**	29.17	12.67	**87.33**		69.67	16.33	14.00	**86.00**
	s.d.	**5.19**	5.08	2.94	**2.94**		8.41	3.56	7.62	**7.62**
C/10	m.	**50.33**	35.17	14.50	**85.50**		**66.17**	19.50	14.33	**85.67**
	s.d.	**8.55**	6.71	3.45	**3.45**		**10.98**	6.35	4.84	**4.84**
C/50	m.	**55.33**	34.33	10.33	89.67		**63.17**	21.00	15.83	**84.17**
	s.d.	**5.43**	7.28	3.78	3.78		**10.63**	4.90	6.62	**6.62**
C/100	m.	**65.17**	29.17	5.67	94.33		73.83	12.67	13.50	86.50
	s.d.	**13.59**	12.59	1.97	1.97		7.57	3.14	7.06	7.06

		Larval morphotypes			
		Normal	**Abnormal**	**Delayed**	**Viable**
FSW	m.	78.17	14.42	7.42	92.58
(Control)	s.d.	4.24	4.48	4.68	4.68

Experiments with *P. miliaris* larvae were performed with FBSW only (Station 250; Station VB8, Table 12). It appeared that the exposure of echinoid larvae to waters from both stations reduced the percentage of normal and viable morphotypes observed after 72 h. The higher reduction was observed after exposition to VB8 water after inverted filtration (intact colonies, Table 12).

The reductions in rates of normal and viable morphotypes of *A. rubens* larvae in response to FBSW were higher than with *P. miliaris* larvae (Station 250; Station VB8, Table 13). The allelopathic effects of those waters were preserved after 5 days storage at -80°C. They were reduced (Station VB8, Table 13) or even almost disappeared (Station 250) after 5 days storage at 14°C. This indicates that the observed allelopathic effect of those natural waters is related to the presence of some colony-derived compound(s). The highest reduction of normal morphotype of *A. rubens* larvae (*i.e.* reduction of 65%) was observed using VB8 water after normal filtration (broken colonies, Table 13). Organic compounds concentrated by *P. globosa* colonies seem to contribute clearly to the assessed allelopathic effect.

Table 13. *Asterias rubens*: Percentages (mean ± s.d.) of each larval morphotype after exposition to filtered waters from station VB8 throughout embryogenesis (72 h). Waters were submitted to larvae in fresh condition or after 5 days at 14±1°C or -80°C. 6 replicates by exposure, 100 larvae scored by replicate. FSW-T0: control in filtered sea water for fresh waters. FSW-T5: control in filtered sea water after 5 days. There were significant differences between the results in bold and the corresponding control (Test performed on normal and viable larvae only; Dunnett, α= 0.05).

		Inverted filtration (Intact colonies)					**Normal filtration (Broken colonies)**			
		Filtered water					**Filtered water**			
Dilution		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Abnormal**	**Delayed**	**Viable**		**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**23.83**	37.33	38.83	**61.17**		**19.00**	36.00	45.00	**55.00**
	s.d.	**5.42**	6.98	7.03	**7.03**		**5.66**	4.15	6.23	**6.23**
C/2	m.	**18.17**	34.33	47.50	**52.50**		**20.67**	38.67	40.67	**59.33**
	s.d.	**6.11**	4.63	7.50	**7.50**		**5.43**	4.46	7.71	**7.71**
C/10	m.	**23.50**	37.50	39.00	**61.00**		**20.67**	42.00	37.33	**62.67**
	s.d.	**6.92**	6.60	3.58	**3.58**		**7.31**	7.24	5.61	**5.61**
C/50	m.	**16.83**	38.33	44.83	**55.17**		**23.83**	39.83	36.33	**63.67**
	s.d.	**2.14**	5.54	6.74	**6.74**		**3.49**	5.38	4.63	**4.63**
C/100	m.	**25.50**	38.67	35.83	**64.17**		**17.67**	40.33	42.00	**58.00**
	s.d.	**3.62**	5.43	2.14	**2.14**		**2.66**	2.50	3.46	**3.46**
		Larval morphotypes								
		Normal	**Abnormal**	**Delayed**	**Viable**					
FSW-T0	m.	41.57	32.83	25.60	74.40					
(Control)	s.d.	4.78	4.11	4.40	4.40					

Table 13 (continued)

Dilution		Filtered water stored at 14°C – Larval morphotypes				Filtered water stored at 14°C – Larval Morphotypes			
		Normal	**Abnormal**	**Delayed**	**Viable**	**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**37.83**	41.67	20.50	79.50	**41.67**	33.83	24.50	75.50
	s.d.	**7.52**	7.37	1.64	1.64	**8.64**	4.40	8.04	8.04
C/2	m.	**42.00**	38.67	19.33	80.67	**32.00**	32.00	36.00	**64.00**
	s.d.	**4.47**	4.18	4.13	4.13	**11.30**	5.37	9.10	**9.10**
C/10	m.	**38.67**	36.50	24.83	75.17	**32.67**	37.50	29.83	**70.17**
	s.d.	**6.50**	5.39	4.71	4.71	**5.92**	5.01	4.36	**4.36**
C/50	m.	**40.50**	36.50	23.00	77.00	**19.67**	53.67	26.67	73.33
	s.d.	**10.80**	7.45	5.93	5.93	**4.84**	7.37	7.97	7.97
C/100	m.	**41.83**	31.17	27.00	**73.00**	**21.17**	50.17	28.67	**71.33**
	s.d.	**7.28**	6.62	7.56	**7.56**	**8.57**	7.11	7.79	**7.79**

Dilution		Filtered water stored at -80°C – Larval morphotypes				Filtered water stored at -80°C – Larval Morphotypes			
		Normal	**Abnormal**	**Delayed**	**Viable**	**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**41.00**	30.67	28.33	**71.67**	44.50	28.33	27.17	**72.83**
	s.d.	**8.10**	5.50	6.80	**6.80**	8.87	5.96	4.22	**4.22**
C/2	m.	**40.17**	35.83	24.00	76.00	**42.00**	31.50	26.50	73.50
	s.d.	**6.79**	4.07	7.87	7.87	**6.96**	5.61	7.69	7.69
C/10	m.	44.00	32.00	24.00	76.00	**41.50**	33.67	24.83	75.17
	s.d.	4.90	5.10	5.14	5.14	**7.61**	6.41	7.22	7.22
C/50	m.	44.67	30.83	24.50	75.50	48.50	27.33	24.17	75.83
	s.d.	6.31	5.71	2.51	2.51	5.32	3.56	2.71	2.71
C/100	m.	**34.33**	38.33	27.33	**72.67**	49.17	28.83	22.00	78.00
	s.d.	**8.69**	4.93	7.34	**7.34**	7.22	4.96	4.82	4.82

		Larval morphotypes			
		Normal	**Abnormal**	**Delayed**	**Viable**
FSW-T5 (Control)	m.	54.06	25.72	20.22	79.78
	s.d.	12.97	8.48	6.19	6.19

1.3.3.3. Toxicity of Skeletonema costatum cultures.

Cultures of the non-colonial diatoms *S. costatum* were used as controls. However, exposure of *A. rubens* larvae to FCSW from *S. costatum* cultures reduced drastically the percentage of both normal and viable larvae (*i.e.* reduction of 75% and 50%, respectively). Organic extracts from the *S. costatum* FCSW or from organic extract performed on filter-collected algal cells induced reductions of both normal and viable larvae. Once again, this allelopathic effect decreased more or less regularly with increasing dilution. It was recently demonstrated that *S. costatum* was able to release an algal toxin very similar to the one released by *Phaeocystis poucheti* (Miralto *et al.* 1999, Ianora *et al.* 2004).

1.3.3.4.Toxicity of Phaeocystis globosa cultures.

FCSW from *P. globosa* cultures which grew under the normal N:P ratio induced a high reduction of the rates of normal and viable larvae (Table 14). At the higher tested concentration in FCSW (*viz.* corresponding to the original concentration of the culture water), the rates of normal larvae were lower when the colonies were broken (reduction of 97.5%, normal filtration) than when preserved (reduction of 81.5%, inverted filtration). Extracts from FCSW or from the colonies presented the same allelopathic properties whichever the filtration performed (Table 14). The FCSWs and the substances extracted from *P. globosa* cultures which grew under low-N:P ratio (5) or high-N:P ratio (100) showed similar profile of response. However, the reduction in normal larvae induced by those FCSW was never as high as that measured in response to FCSW form culture which grew under the normal N:P ratio. Alterations of N:P ratio during the growing period of the micro-alga do not stimulate the production of compounds with allelopatic properties.

Table 14. *Asterias rubens*: Percentages (mean ± s.d.) of each larval morphotype after exposition to filtered waters and organic extracts from cultures of *Phaeocystis globosa* (N:P ratio: 16) throughout embryogenesis (72 h). **ASW**: control in artificial sea water. There were significant differences between the results in bold and the corresponding control (Test performed on normal and viable larvae only; Dunnett, α= 0.05).

		Inverted filtration (Intact colonies)					**Normal filtration (Broken colonies)**			
		Filtered water					**Filtered water**			
Dilution		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Abnormal**	**Delayed**	**Viable**		**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**6.17**	38.67	55.17	**44.83**		**0.83**	36.83	62.33	**37.67**
	s.d.	**3.54**	6.95	7.31	**7.31**		**1.60**	8.50	9.16	**9.16**
C/2	m.	**13.83**	42.33	43.83	**56.17**		**4.83**	40.17	55.00	**45.00**
	s.d.	**2.64**	1.51	3.87	**3.87**		**2.93**	2.56	4.00	**4.00**
C/10	m.	**24.67**	40.67	34.67	**65.33**		**14.17**	39.67	46.17	**53.83**
	s.d.	**1.86**	2.07	1.75	**1.75**		**1.60**	3.20	4.75	**4.75**
C/50	m.	29.67	42.00	28.33	71.67		**17.50**	36.83	45.67	**54.33**
	s.d.	5.13	9.14	5.28	5.28		**2.95**	2.14	4.23	**4.23**
C/100	m.	30.17	40.67	29.17	70.83		**18.17**	39.17	42.67	**57.33**
	s.d.	5.56	3.01	4.71	4.71		**2.71**	6.43	5.32	**5.32**

Table 14 (continued)

		Extracts from the filtered water					Extracts from the filtered water			
Dilution		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Abnormal**	**Delayed**	**Viable**		**Normal**	**Abnormal**	**Delayed**	**Viable**
C	m.	**28.83**	39.00	32.17	67.83		28.67	34.50	36.83	**63.17**
	s.d.	**4.12**	2.61	5.42	5.42		3.50	4.89	3.31	**3.31**
C/2	m.	**29.00**	39.33	31.67	68.33		**27.17**	34.67	38.17	**61.83**
	s.d.	**3.29**	3.01	3.93	3.93		**9.02**	6.19	4.58	**4.58**
C/5	m.	**28.33**	32.50	39.17	**60.83**		**25.33**	35.67	39.00	**61.00**
	s.d.	**1.37**	4.55	4.17	**4.17**		**5.16**	5.13	3.58	**3.58**
C/10	m.	31.33	35.83	32.83	67.17		**26.17**	32.17	41.67	**58.33**
	s.d.	5.79	5.85	4.17	4.17		**5.78**	4.12	4.93	**4.93**
		Extracts from cellular material					**Extracts from cellular material**			
Dilution		**Larval morphotypes**					**Larval Morphotypes**			
		Normal	**Abnormal**	**Delayed**	**Viable**		**Normal**	**Abnormal**	**Delayed**	**Viable**
2.5C	m.	**44.00**	31.83	24.17	75.83		**23.17**	38.00	38.83	**61.17**
	s.d.	**4.10**	4.71	4.12	4.12		**7.47**	5.73	10.03	**10.03**
C	m.	35.83	35.67	28.50	71.50		**25.50**	35.67	38.83	**61.17**
	s.d.	6.11	4.72	5.05	5.05		**7.06**	5.89	8.23	**8.23**
C/2	m.	**40.50**	33.33	26.17	73.83		**24.50**	29.83	45.67	**54.33**
	s.d.	**3.39**	4.46	4.62	4.62		**6.38**	5.71	10.01	**10.01**
C/5	m.	35.33	36.83	27.83	72.17		28.17	31.67	40.17	**59.83**
	s.d.	6.92	3.54	3.92	3.92		5.04	4.27	7.39	**7.39**
C/10	m.	30.83	40.17	29.00	71.00		29.00	32.50	38.50	**61.50**
	s.d.	5.71	5.98	6.66	6.66		3.63	5.96	5.96	**5.96**
		Larval morphotypes								
		Normal	**Abnormal**	**Delayed**	**Viable**					
ASW (Control)	m.	33.48	38.12	28.40	71.60					
	s.d.	4.48	5.15	5.52	5.52					

P. globosa appears to release water soluble compounds able to interfere with the development of echinoderm larvae. These compounds are produced in the water column but also concentrate within the colonies. They could be massively released in the environment when *P. globosa* colonies become senescent. In the North Sea, this period of the vital cycle of *P. globosa* corresponds to a peak of abundance of *A. rubens* larvae in the plankton (Rousseau *et al.* 1994). Our experiments indicated that larvae of *A. rubens* were particularly sensitive to these algal compounds. The influence of *Phaeocystis* algae on the perpetuation of local starfish populations should not be underestimated and should be the subject of long-term studies. Moreover, our results also showed that other common algal species could produce compounds with allelopathic properties (*S. costatum* for example).

1.4. Juvenile growth

1.4.1. Characterisation of study sites

The study locations differed in term of substratum, wave exposure, salinity conditions and emersion frequency for the starfish populations (Table 1). In Breskens, Scheldt estuary, the area is sheltered from wave action. The population was submitted to large daily and yearly salinity variations. Starfish were found under stones on mud, no mussel bed was present. The Knokke population, first studied by Jangoux & Vloebergh (1973), was situated in the Scheldt plume, where salinity variations were less pronounced than at Breskens. Starfish were found on a breakwater, covered by a mussel bed, on an exposed sandy beach. In these two locations starfish were more often emerged than in Aber and Ambleteuse. In the latter, the populations were exposed to rather similar environmental conditions: they were living in a marine area on an exposed shore where an extended mussel bed that continues in the subtidal zone was present. However in Aber, starfish were not found on the mussel bed but on a small accumulation of pebbles and small boulders at the rock-sediment limit. Only larger individuals were collected at the base of the mussel bed (few meters away). In both populations no starfish could be collected if the low tide water level was above 1 meter.

Food available to recruits in each location was highly variable at a small scale as evidence by very high standard deviations assorted with most values. Total biomasses of available food did not differ significantly among locations (p ANOVA = 0.074) and seasons (p ANOVA = 0.645). Biomasses of small mussels showed a slightly significant interaction term (p ANOVA = 0.021) but the Tuckey multiple mean comparison test failed to detect significant pairwise comparison. The largest pairwise differences were noticed for the August sampling, small mussel biomasses being much higher at Breskens than at Aber and Knokke. Barnacle biomasses did significantly differ from location to location (p ANOVA = 0.022); the interaction term was of a low significance (p ANOVA = 0.049): the barnacle biomass at Breskens in August was significantly higher than that at Aber in both seasons.

1.4.1.1. Metal contamination

Level of metals contaminations in the pyloric caeca and body wall of adult starfish from the different populations are available in the "Belgian Marine Data Centre" (http://www.mumm.ac.be/datacentre/). Starfish from Ambleteuse appeared as the less contaminated by metals while those from Knokke and Breskens appeared as the most contaminated. A contamination by zinc of the pyloric caeca and by cadmium of the body wall of Aber starfish was also evidenced.

1.4.1.2. PCDD/F and c-PCB concentrations

Complete data sets may be found in the "Belgian Marine Data Centre" (http://www.mumm.ac.be/datacentre/) and in Danis et al (2006).

Mussels (*Mytilus edulis*) were analyzed for their content in PCDDs, PCDFs and co-PCBs. No significant differences were found between DLC levels measured in mussels from the different sampling stations ($p_{Kruskal-Wallis}>0.1$), but some trends could be highlighted: mussels from Wenduine tended to be more contaminated by PCDFs and PCDDs, while higher levels of co-PCBs were measured in mussels from those from Ambleteuse.

Total TEQ (pg TEQ g^{-1} lipids) measured in mussels were remarkably constant, with values ranging from 124 pg TEQ g^{-1} lipids in Nieuwpoort to 159 pg TEQ g^{-1} lipids in Knokke. Most of the TEQ was attributable to co-PCBs. OCDD was the most abundant among dioxins, TCDF was the most abundant of furans and PCB 77 displayed the highest concentration among co-PCBs. When considering all contaminants together, PCB 77 was always the most concentrated congener, whichever station was considered (proportion ranged from 60 to 67%, with a low variability) (Table 2B).

PCDD/F and c-PCB concentrations in sea stars are shown in Table 15 (sea star samples from Nieuwpoort were lost during DLC analysis). The situation was more contrasted than in the case of sediments and mussels analyses, as significant differences were found between $\sum_{10}$PCDFs levels measured in sea stars from the different stations, ($0.01<p_{Kruskal-Wallis}<0.005$). Sea stars from Ambleteuse were less contaminated by PCDDs, while sea stars from Oostende, Knokke and Wenduine displayed very close values. The same ranking of the sampling stations was observed for furans, and higher concentrations were measured in sea stars from Knokke. Sea stars from Knokke displayed the lowest co-PCBs concentrations, while those from Ambleteuse, Oostende and Wenduine displayed similar values for this class of contaminant. PCDD and PCDF concentrations were lower than in mussels, while co-PCB levels were higher than in mussels. OCDD was the most concentrated among dioxins, TCDF was the most concentrated among furans and PCB 77 was the major congener among co-PCBs. When considering all contaminants together, PCB 77 was always predominant, but its degree of predominance was lower in Knokke (55%) than in the other sampling stations (values ranging from 80 to 88%).

The contribution of the different classes of contaminants to the total toxicity in sea stars displayed the same scheme as in mussels. The major part of toxicity was due to co-PCBs, this trend being even more manifest than in the case of mussels.

In order to assess the possible biomagnification of the different congeners, bioaccumulation factors (BAF; ratio between mean concentration in sea stars and mean concentration in mussels) were calculated, for each sampling station (Fig 2). Coplanar PCBs were found to be biomagnified in almost all cases (BAFs ranging

from 0.67 to 3.01), whereas the other contaminant classes usually displayed BAF values ≤1.

Table 15: Concentrations of DLCs measured in sea stars (*Asterias rubens* ; Mean±SD ; pg g^{-1} lipids) from the southern North Sea and the English Channel. ND = not detected; values <limit of quantification (LOQ) are attributed a fixed value (upper bound) ; values in bold represent the major congener, for each sampling station.

	Station			
	Ambleteuse (n=3)	Oostende (n=3)	Wenduine (n=2)	Knokke (n=2)
TCDD	0.82 ± 0.05	7.73 ± 0.47	7.87	11.8
PeCDD	2.06	7.79 ± 0.23	7.96	7.66
$HxCDD_1$	1.03	2.72 ± 0.58	3.41	2.97
$HxCDD_2$	3.21	11.7 ± 0.84	12.3	10.9
$HxCDD_3$	1.51 ± 0.02	4.62 ± 0.48	5.16	4.32
HpCDD	10.8 ± 0.25	32.9 ± 2.55	31.9	30.6
OCDD	**30.3 ± 0.57**	**78.2 ± 4.95**	**75.1**	**69.9**
$\sum_7$PCDDs	47.3 ± 5.68	146 ± 5.28	143.7	138
TCDF	**70.3 ± 6.37**	**124 ± 14.9**	**134.9**	**156**
$PeCDF_1$	ND	0.93 ± 0.30	ND	1.50
$PeCDF_2$	6.75	22.0 ± 3.83	21.4	44.4
$HxCDF_1$	0.58	0.43 ± 0.19	0.74	0.66
$HxCDF_2$	ND	ND	ND	0.47
$HxCDF_3$	ND	ND	ND	ND
$HxCDF_4$	0.57	3.50 ± 0.31	2.83	4.27
$HpCDF_1$	ND	3.61	ND	ND
$HpCDF_2$	ND	ND	ND	ND
OCDF	ND	ND	ND	ND
$\sum_{10}$PCDFs	75.5 ± 0.84	152 ± 17.6	159	207
$\sum_{17}$PCDD/Fs	123 ± 0.27	298 ± 16.7	303	345
PCB 77	**7280 ± 1590**	**7290 ± 1460**	**6360**	**2430**
PCB 81	355 ± 20.9	96.1 ± 20.4	229	190
PCB 126	552 ± 59.5	1030 ± 141	988	1360
PCB 169	ND	92.7 ± 2.40	95.4	131
$\sum_4$cPCB	8070 ± 1673	8440 ± 1465	7670	4110

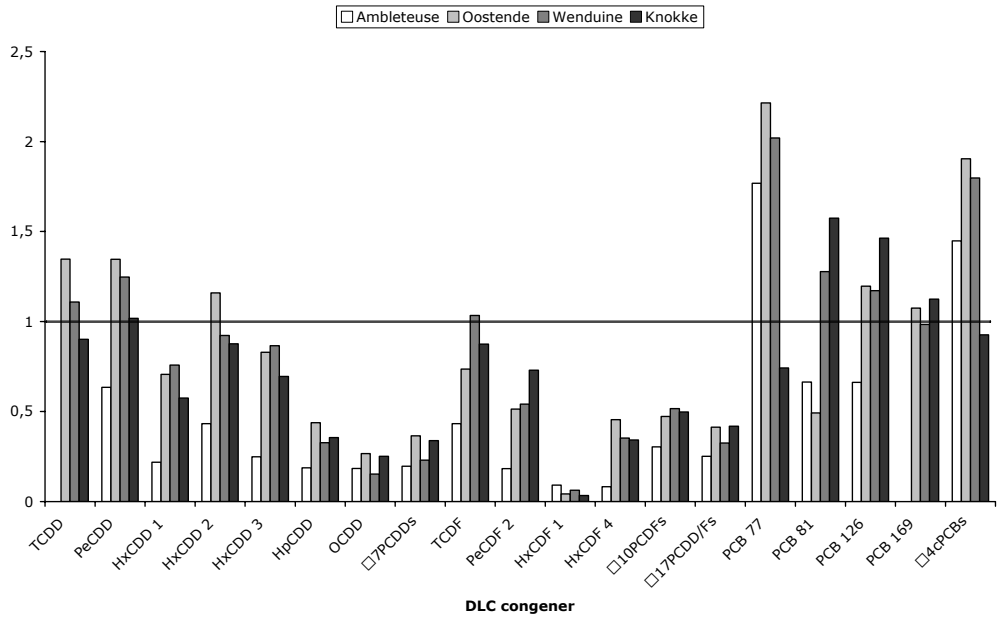


Figure 2: Bioaccumulation factors (ratio between the mean concentration measured in sea stars and the mean concentration measured in mussels) calculated for the different congeners, in each sampling station

1.4.1.3. CYP1A IPP induction in sea stars

The induction of a CYP1A IPP was measured using competitive ELISA in sea stars collected in the different stations (Fig. 3). Significant differences ($p_{Kruskal-Wallis} \leq 0.0001$) were found between the different stations: sea stars from Knokke displayed the highest CYP1A IPP levels. The lowest induction values were recorded in sea stars from Ambleteuse. Values measured in sea stars from Oostende, Nieuwpoort and Wenduine were intermediate.

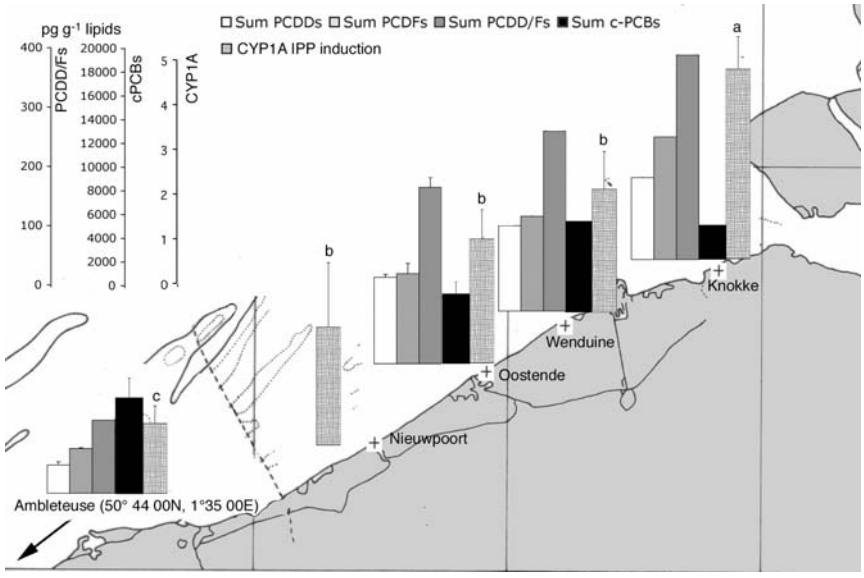


Figure 3: Contaminant levels (mean±SD, pg g^{-1} lipids), and CYP1A immunopositive protein induction (mean±SD, n=9) measured in sea stars (*Asterias rubens*) from the different sampling stations.

Regressions were calculated between mean CYP1A IPP induction and DLC concentrations or TEQ in corresponding sea star pools. When considering concentrations, significant regressions were found between CYP1A IPP induction and $\sum_{10}$PCDFs or $\sum_{17}$PCDD/Fs concentrations measured in sea stars. Determination coefficients respectively reached 0.68 (p=0.002) and 0.63 (p=0.004). Highly significant regressions were also found between CYP1A IPP induction and TEQs for all contaminant classes (Fig 4). Determination coefficients ranged between 0.56 (CYP1A IPP induction as a function of $\sum_{7}$PCDDs TEQ in sea stars) and 0.79 (CYP1A IPP induction as a function of $\sum_{10}$PCDFs TEQ in sea stars).

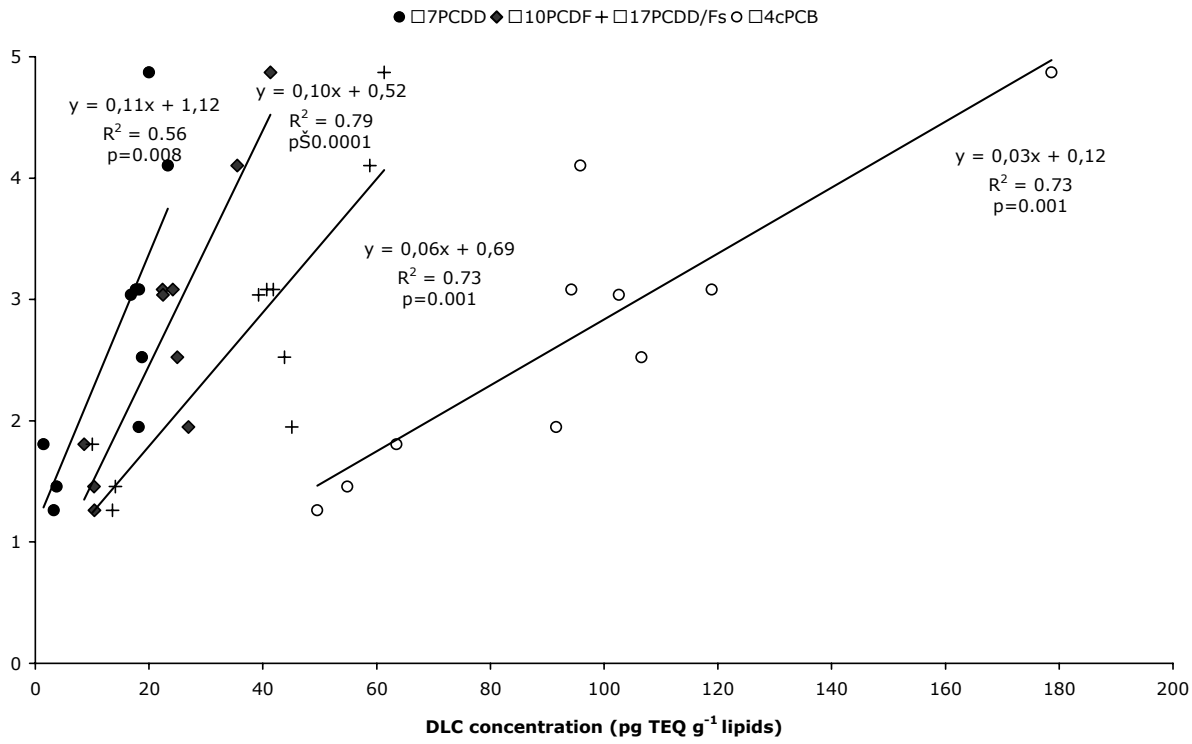


Figure 4: Regressions between mean CYP1A IPP induction (Time fold) and TEQs values of the different DLC classes (pg TEQ g^{-1} lipids) determined in sea star pyloric caeca. R^2: corrected determination coefficient.

1.4.2. Population dynamics

Size frequency distributions were observed during 2 or 3 years in the four studied populations. Cohorts were tentatively delimited by modal analysis. Growth of juveniles was determined using the mean size of the fitted modes. Size frequency distributions and growth of juveniles strongly differed between the Aber population and the 3 Northern populations (Fig. 5).

The 3 Northern populations showed a well-defined annual recruitment (sensu Ebert 1983) which was first observed between June and August of each year. The proportion of starfish represented by the fitted normal mode was used as an indicator of recruitment intensity. As part of the recruits could be cryptic or immerged and

therefore undersampled, recruitment intensity was assessed after it "stabilised" in proportion i.e. 2-3 months after first observation. Recruitment intensity showed annual variations within each population (Table 16). In general, recruits were always found together with adults except in Knokke, where they first appeared on the lower boulders of the breakwater and then, within one or two months, migrated to the area with adults.

During the first 3-5 months after recruitment the growth rate was high (Fig. 5). It differed in duration among populations and between years (Table 16). Generally, growth of juveniles in Ambleteuse was significantly higher than at Knokke and Breskens. This fast growth period was followed by a slow growth period, which varied in length and rate of growth between populations and years (Fig. 5). In Breskens, after Aug. 01 no growth was observed. In Knokke, the growth stopped at the beginning of fall (Oct.00 and Sept. 01) and appeared to resume in January. A few months after growth resumed, cohorts of juveniles and of older starfish merged to form a single mode. No estimation of the growth rate during this second growth period due to its short duration (2-3 months, both years). In Ambleteuse, no cessation of growth was observed, rather growth continued at a low rate until the following summer ($n = 5$, $p = 0.040$, slope = 0.075 ± 0.021, $R^2 = 0.802$). Juvenile and older starfish cohorts merged into a single mode about one year after recruitment (Fig. 5). The larger starfish present in the field were most of the time represented by only one mode even in Ambleteuse where this group could account for 70% of the population. The attempts made to decompose this group in more than one mode never succeeded in a logical pattern that could be followed during several months. The growth rates of the largest starfish were low and their average mean size was characteristic of each population: 85 ± 8 mm in Ambleteuse, 42 ± 5 mm in Knokke and 27 ± 6 mm in Breskens (Fig. 5). In Ambleteuse, a recapture trial on large starfish was attempted in September 2001. One month later, only 2% of the marked individuals were recaptured in the intertidal area.

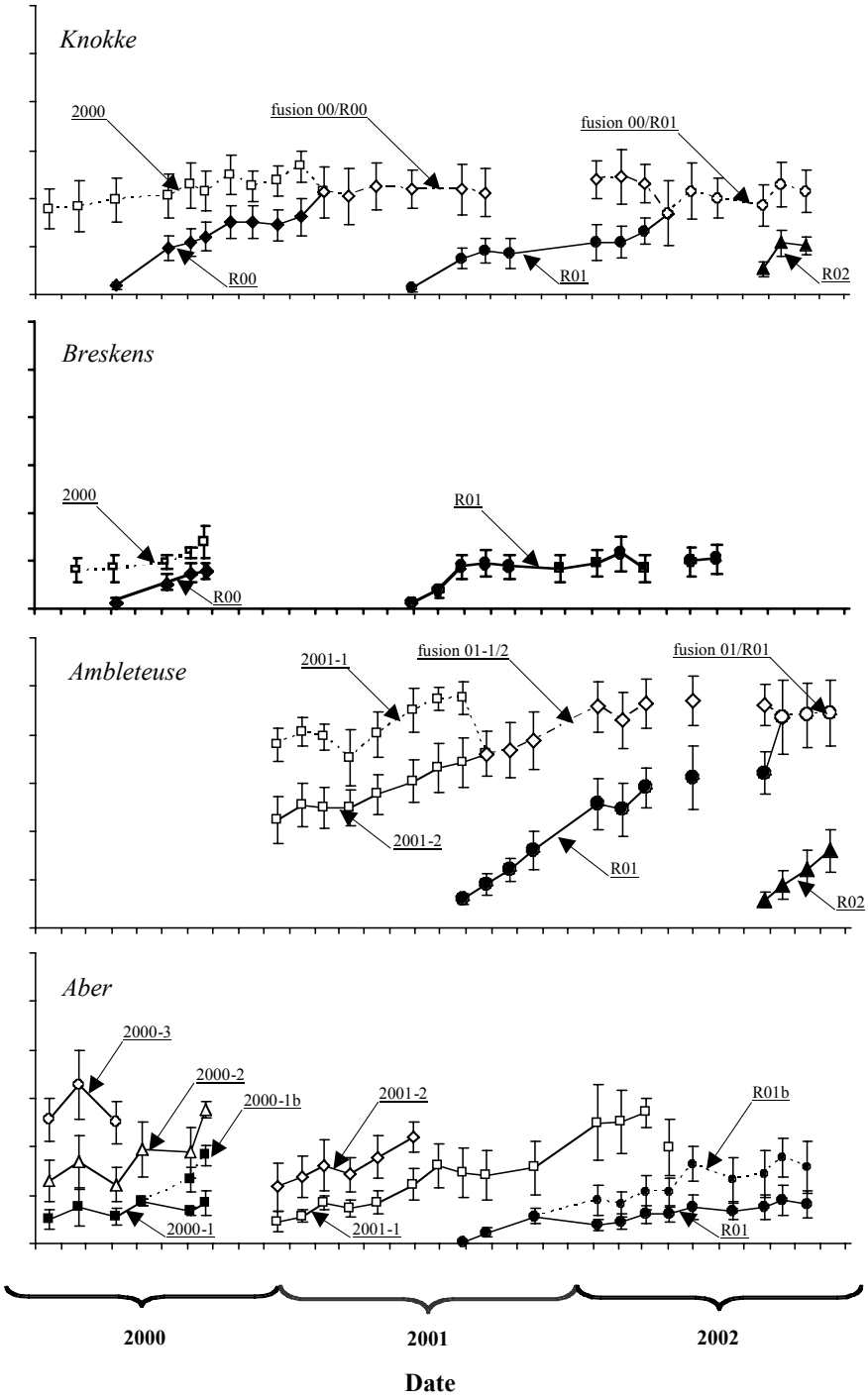


Figure 5. Temporal changes in the mean size (ray length in mm) of the different cohorts of starfish from April 2000 to November 2002 from fitted normal modes estimated by MIX3.0 (mean ± standard deviation). Cohorts are named either from their date of first observation (e.g. Aber 2000-1 is the cohort of the smallest starfish collected in 2000) or from the year of observed recruitment (e.g. Knokke R00 is the cohort of starfish which recruited in 2000). When one cohort splits into 2 distinct normal modes, the second mode is named b (e.g. Aber 2000-1b is issued from 2000-1). If 2 cohorts merged into one normal mode, this normal mode is called "fusion" (e.g. in Knokke, fusion00/R00 is the result of the fusion of the cohorts 2000 and R00).

Table 16: Characteristics of the recruitment and parameters of the fast growth period as modeled by linear regression of the mean size of the juvenile mode against time. Intensity is the proportion of juveniles in the population. Slopes sharing the same superscript are not significantly different (ANCOVA comparison with α = 0.05), non-significant regressions were not ranked

Population	Aber	Ambleteuse		Knokke			Breskens	
Year	2001	2001	2002	2000	2001	2002	2000	2001
Intensity	50%	26%	84%	89%	64%	38%	79%	100%
First observation	August	August	August	June	June	June	June	June
End of fast growth period	January	January	?	November	September	September	September	September
n	4	5	4	5	3	3	3	3
Slope ($mm.days^{-1}$) ± standard error	0.107^{c} ± 0.008	0.246^{a} ± 0.006	0.253^{a} ± 0.007	0.184^{b} ± 0.017	0.180 ± 0.022	0.176 ± 0.148	$0.128^{b,c}$ ± 0.005	0.239 ± 0.060
p (regression)	0.006	0.000	0.001	0.002	0.077	0.446	0.026	0.156
R^2	0.986	0.998	0.999	0.976	0.986	0.585	0.998	0.941
Growth rate ($mm.month^{-1}$)	3.21	7.38	7.59	5.52	5.40	5.28	3.8	7.2

In Aber, the population dynamics appeared different from those of the other studied populations. Small starfish, between 10 and 20 mm in ray length, were always present on the shore and population structure was less stable through time (the small starfish mode split in late summer 2000 and the population structure change during fall 2000). Only one recruitment was obvious (Aug. 01). It was composed of recruits, all measuring 0.4 mm in ray length, that occurred under boulders. During winter 2001-2002, this juvenile mode split into 2 distinct modes: one of small starfish (R01 on Fig. 3) that kept the same mean size troughout 2002 and one of starfish (R01b) that continued to grow. The growth rate of the 2001 recruits was assessed before the mode split into 2 (Table 4). It was not statistically different from that of 2000 recruits in Breskens, but was smaller than that of Knokke recruits (2000) and Ambleteuse recruits (2001 and 2002). In Aber, the larger starfish modes (2000-2, 2000-3, 2001-1) disappeared from the intertidal location after they reached a size greater than 50 mm ray (i.e. 56 ± 9, 56 ± 3 and 52 ± 2 respectively).

1.4.3. LABORATORY EXPERIMENTS

1.4.3.1. Influence of food supply on juvenile growth

Starfish fed *ad libitum* consumed ½ mussel $starfish^{-1}$ day^{-1} all through the experiment; "half satiety" diet was therefore set at ¼ mussel $starfish^{-1}$ day^{-1}. Starfish fed *ad libitum* showed a higher growth rate. Starfish provided with "Aber food supply" (rock scrapping from the collection site) split into 2 groups, which were tentatively

demarcated by modal analysis: a small proportion (about 25%) showed a fast growth and a large one grew slowly. The growth rate of the former was significantly higher than that of starfish fed *ad libitum* with mussels. The growth rate of the latter was similar to that of starfish fed at "half satiety".

1.4.3.2. Emersion influence on juvenile growth

No difference in size was observed between starfish submitted to emersion and individual always immersed (mean size was 33 ± 7 mm under tidal emersion and 36 ± 7 mm under continuous immersion conditions, p ANOVA = 0,070). Growth rates were 0.115 ± 0.010 mm.day^{-1} for starfish submitted to tidal emersion (n = 6, p < 10^{-3}, R^2 = 0.97) and 0.135 ± 0.012 mm.day^{-1} in immersion condition (n = 6, p < 10^{-3}, R^2 = 0.97). They were not statistically different (ANCOVA comparison with α = 0.05). Feeding rate was greater in the immersion treatment (feeding rate was 1.79 ± 0.89 mussel starfish^{-1} day^{-1} under tidal emersion and 2.51 ± 0.80 mussel starfish^{-1} day^{-1} under continuous immersion conditions, p ANOVA < 10^{-3}). Mean righting time was 167 ± 160 seconds for starfish submitted to emersion (n=59) and 166 ± 158 seconds for starfish continuously immersed (n=57). Emersion had no influence on the righting time (p Kruskal-Wallis = 0.57).

1.4.3.3. Influence of salinity variations on juvenile growth

In this experiment, the growth rate could not be assessed because the experiment duration was too short (15 days). Individual deltas of size (= mean size of the 5 arms at the end minus mean size of the 5 arms at the beginning) were calculated. The highest increase in size took place for treatment with a constant salinity of 34 PSU (OS and CS34) and the lowest for starfish exposed permanently or intermittently at 26 PSU (CS26 and VS26). Feeding rates calculated over 24 hours were lower for starfish exposed permanently or intermittently at 26 PSU (CS26 and VS26). Those calculated over 14 h did not differ except that they were lower for starfish permanently kept at 26 PSU (CS26) compared to those exposed intermittently to 30 PSU (VS30). Those calculated over 10 h were lower for starfish exposed intermittently at 26 PSU (VS26) and higher for starfish exposed to a constant salinity level (OS, CS34 and CS26). There were no significant differences between the open system and the closed system with constant exposure to 34 PSU in terms of growth or feeding rates, indicating that enclosed system did not introduce a bias. Salinity had no influence on the righting time (n = 100, p Kruskal-Wallis = 0.38).

1.4.3.4. Influence of sea water temperature on juvenile growth

No difference in size was observed at the end of the experiment between starfish reared at the 3 temperatures (mean sizes were 41 mm ± 7 at 8-9°C, 44 mm ± 6 at 13°C and 42 mm ± 6 at 16-17°C; p ANOVA = 0.26, n = 9). Growth rates were 0.148 ±

0.018 mm.day^{-1} at 8-9°C (n = 5, p regression = 0.004, R^2 = 0.96), 0.168 ± 0.018 mm.day^{-}1 at 13°C (n = 5, p regression = 0.001, R^2 = 0.97) and 0.151 ± 0.018 mm.day^{-1} at 16-17°C (n = 5, p regression = 0.004, R^2 = 0.96) ; they were not statistically different (ANCOVA comparison with α = 0.05). No change in the growth rates was observed after removal of half of the number of starfish in the middle of the experiment.

1.4.4. Discussion

We only occasionnaly succeeded in decomposing size-frequency distributions into more than 2 modes. In most cases, the juvenile mode fused over time with an older mode, concomitantly with a decrease in the latter. When a third mode was found, it was only present for a short period or in the particular condition of the Aber (see below). This strongly differed from previous studies on *Asterias rubens* where several modes (up to seven, Guillou 1983), all attributed to one year class, were identified from the size frequency distributions. This discrepancy can be explained by the difference in the methods used. Nichols & Barker (1984) arbitrarily chose the size at age distribution postulating the occurrence of 3 age classes of fixed size. Nauen (1978) determined modes visually. Finally, Guillou and collaborator's analysis was based on Bhattacharya graphical determination of modes (Guillou 1979, 1983, Guillou & Guillaumin 1984), which tends to generate supernumerary modes (Grant et al. 1987, Macdonald & Pitcher 1979). In the present study, we used modal analysis, which is a more objective method (Macdonald & Pitcher 1979). As it did not allow us to distinguish adult cohorts from each other, we could not assess the age and lifespan of adult starfish. This is probably due to highly variable individual growth rates and to mortality or migration of larger starfish (see below). Consequently, we focused on the growth pattern during the first year after recruitment and did not attempt to fit any growth model to our data. However, it is noteworthy that the maximal modal size reached by the adult mode differed between populations. It appeared to be stable through time in each population and therefore we considered it as characteristic of the population.

Observations made on the 3 northern populations were consistent with the previously described life cycle of *A. rubens* in the North Atlantic. Spawning took place in April-May (Joly et al. 2003) and recruitment occured between June and August. In the Aber population, only one recruitment event could be clearly observed. It took place in August 2001. The delay of recruitment compared to the northern populations has to be related to the delay in spawning, which happens later in Aber (Joly et al. 2003). Recruitment was followed first by a period of fast growth and second by a period of slow growth, or even cessation of growth. The duration of these periods varied between populations and this allowed us to distinguish different scenarios, that we have also recognized in previous studies (Table 6).

In Ambleteuse, juveniles showed the highest monthly growth rates ever reported for *A. rubens* and maximal modal size was elevated. In this location, environmental conditions (abundant food supply, marine salinities and low emersion frequency) allowed optimum growth. Moreover, movement of starfish towards the subtidal area (as indicated by the recapture trial) could protect them against environmental stress.

In Knokke, growth just after recruitment was elevated but slower than at Ambleteuse. Growth stopped during winter months (Table 6) as previously observed in other intertidal populations (Guillou 1979, Nichols & Barker 1984) and maximal modal size was small. All this was likely due to less favorable conditions which were enhanced during winter months: high emersion frequency and related stress (desiccation, rainfalls…) and salinity levels variation which can significantly reduce juvenile growth (see below). Migrations of starfish to a subtidal refuge appeared unlikely because of the lack of hard substrata in the vicinity of the breakwater. In our experiment, influence of salinity on juvenile growth was evident. Constant or intermittent exposure to a lower salinity resulted in a decreased growth linked to a reduced food intake as compared to starfish constantly kept at 34 PSU. This was also elicited, even if less pronounced, by intermittent exposure to 30 PSU as may happen in Knokke. On the other hand, emersion influence on juvenile growth was not obvious. Under emersion/immersion conditions, the feeding rate decreased; the starfish were not observed feeding during emersion periods, but the immersion periods were long enough to allow the growth rate to be equivalent to that of starfish reared under permanent immersion condition. However, our experimental design did not mimic the stresses to which starfish can be submitted when emersed in the field (high temperature, dessication or rainfall). Our data only allowed us to conclude that emersion alone, and its effect on foraging time, has no direct effect on juvenile growth when external temperature is low (i.e. 4°C) and close to seawater temperature (i.e. 6°C).

In Breskens juveniles showed a very short period of fast growth followed by a protracted period of very slow growth (2001) or population decline (2000) due to a severe food shortage (lack of mussel recruitment). Growth did not resume in spring and the starfish never reached the size of sexual maturity (35mm, Joly et al. 2003). Estuarine conditions with important salinity variations limited growth (as evidenced in the laboratory experiment). Moreover, dwarfism has already been reported for populations of *A. rubens* living in brackish water (Kowalski 1955). In Aber, during their first winter, the majority of juveniles ceased to grow and remained below the size of sexual maturity for a protracted period, even if some juveniles went on growing. Nauen (1978) has already observed such a permanent stock of small starfish said to be in "waiting stage" (i.e. "waiting for a free seat in the ecological niche"). Mortality or migration of the largest starfish allowed a few individuals to grow, which in return, monopolised the food supply and limited the growth of the remaining

juveniles (waiting individuals). This "waiting stage" was also evidenced whilst testing the influence of food supply on juvenile growth. Indeed, most juveniles provided with Aber food supply showed a low growth rate while only a few of them were able to grow rapidly. Growth rate of the former was similar to that of juveniles fed at "half satiety". Both were lower than that of individuals fed *ad libitum* with mussel indicating that they were food limited. In Aber, wave movements confined starfish in the boulder area where they are submitted to a considerable stress due to inadequate food supply, Disappearance of large individuals was likely due to migration to the subtidal zone as discussed by Guillou (1979).

Expression of the CYP1A immuno-positive protein is a biomarker of organic contamination by dioxin-like compound or polycyclic aromatic hydrocarbon (Bucheli & Fent 1995, den Besten 1998). Its induction in starfish in prespawning stage is similar in the Northern populations and lower in Aber. Starfish from Knokke and Breskens were more contaminated by metals than those from Ambleteuse and Aber. This is linked with the pollution state of the North Sea and of the Scheldt estuary in particular (OSPAR 2000). However, starfish in Aber presented high level of zinc in their pyloric caeca probably due to contamination by zinc-containing manure. The impact of these contaminations in populations is difficult to assess. To detect pollution effects between different populations, other environmental factors have to be comparable. In Aber, juvenile growth is controlled by low food availability, which might hide pollution effects on growth. The Ambleteuse population appears as a good reference point, with background contamination by metals and unlimited juvenile growth. Juvenile growth rates and maximal modal size are higher in Ambleteuse than in Knokke and in Breskens. The main differences between Breskens and Ambleteuse locations are salinity, food quality and pollution. Discrepancies observed between Ambleteuse and Knokke can be explained by the interaction of several "natural" factors, emersion, density and salinity. However, pollution can not be excluded even if "natural" factors alone may account for the observed effects.

1.5. RNA/DNA ratio in adults

The RNA/DNA ratios measured in *A. rubens* body wall showed no significant differences between gender (p=0.36) and the four stations (p=0.61) (detailed results may be found in Catarino et al 2008). 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. However, this was highly dependent on a few possible outliers. When these four values (on a total of 101) were removed from the analysis, there was no more significant difference (p_{gender}=0.92; $p_{station}$=0.19; $p_{interaction}$=0.51). The RNA/DNA values obtained in *E.*

acutus body wall, showed no significant differences between genders and the four stations (p=0.70 for both factors).

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 RNA/DNA ratios and Cd, Pb, and Cu concentrations occurred (p=0.53). However, a significant, but weak, negative relationship between Zn concentration and RNA/DNA ratio was evidenced (p=0.01; R^2=0.072; y=123.59-2.39x).

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 (Melzner at al 2005, Liyana-Pathirana et al 2002, Lannig et al 2006) . Exposure of fish and invertebrates to stress factors, both in laboratory and field conditions, reduced this ratio due to a decrease in RNA content. However, Lannig et al. (2006) reported an increase of RNA/DNA in oysters experimentally exposed to Cd. This was attributed to the production of proteins associated with metal detoxification. Furthermore, 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 the present study results, the RNA/DNA ratio did not differ in the body wall of both *A. rubens* and *E. acutus* from different stations. This could be due to the lower metabolism of this compartment compared to digestive organs, for instance. However, male starfish from S1 showed a significantly higher RNA/DNA ratio in pyloric caeca. This was mainly due to lower DNA concentrations (although they were not significantly lower; data not shown). Lower DNA content has been reported for fish that had been exposed to toxicants, thus affecting RNA/DNA ratio. The lower values were attributed to a reduction in cell multiplication capability, hence mitosis, rather than a cell enlargement (Barron & Adelman 1984). On the other hand, the observed values may also be due to natural variability. The DNA and RNA contents of this body compartment change according to season, that is, to reproductive cycle (Van der Plas & Voogt 1982). Therefore, the individuals in which low levels of DNA were observed might had been in a different maturity stage. Individuals with low levels of DNA, reflecting a high RNA/DNA ratio, were observed in more than one station, but always in very low number (two individuals in S1, one in S3 and one in S4). Furthermore, this difference in RNA/DNA ratio was dependent on a few questionable samples that, when removed from analysis, led to no significant effect. 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 this organ. These facts strongly suggest that RNA/DNA ratio in this starfish of the Sørfjord is not affected by field metal contamination.

2. Bivalves

2.1. Spatial trends

Results showed that sites near to the sea had significantly lower concentrations of all the heavy metals studied. In the cases of Cd, Ni, and Zn, concentrations at Hansweert in the upper part of the estuary at salinity 15-19 ppt (due to tidal cycle), were about an order of magnitude higher compared to Westkapelle on the sea-side at salinity 34 ppt. When the WS and ES were compared, 2-way ANOVA showed a strong effect ($P<0.001$) of *site* on metal concentrations in mussels. Values in the WS were much higher than in the relatively less polluted ES. In the case of cadmium, mussels in the WS had concentrations about 10 times higher than in the ES. Other metals (Co, Cr, Mn, Ni, Pb & Zn), concentrations in the ES were at least two times lower than in the WS (The complete data set can be found in Mubiana et al 2005).

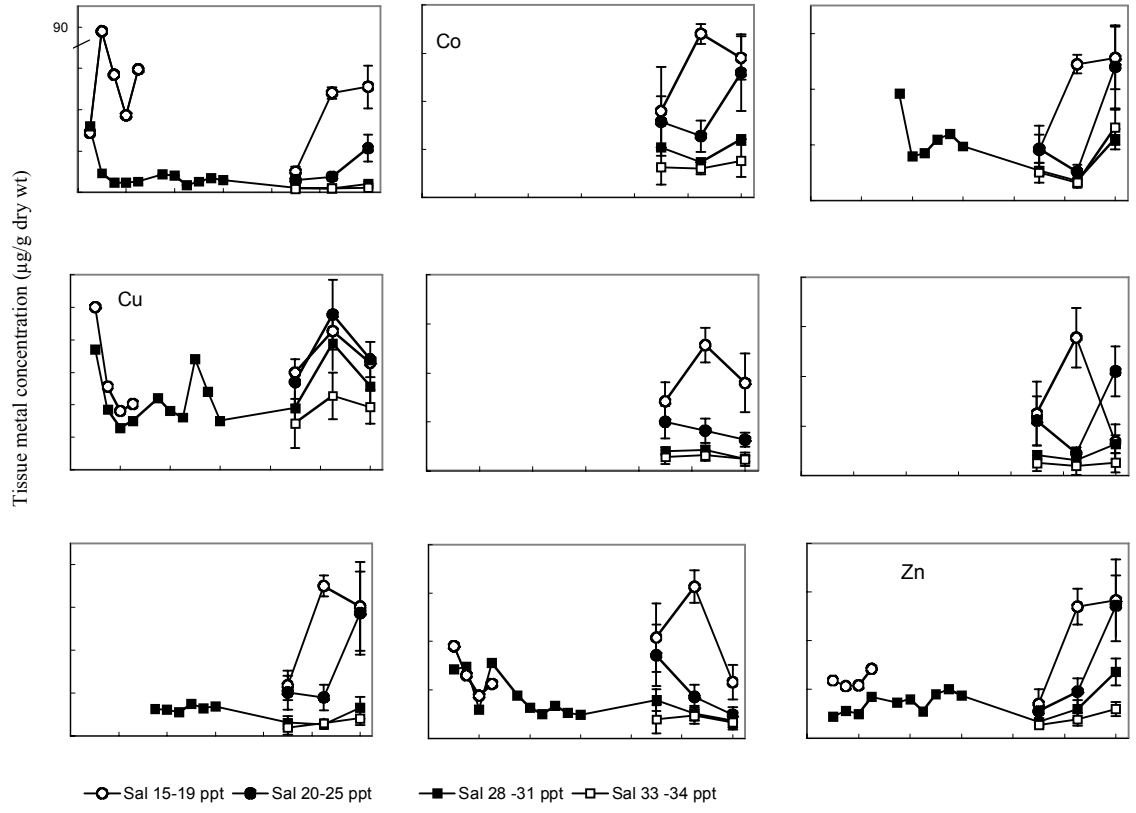


Figure 6: Evolution of metal concentrations in mussel tissues (µg/g) from Western and Eastern Scheldt (The Netherlands). 1979-1992 data was obtained from JMP of the Netherlands and are pooled values of 50 individuals while 1996-2002 values are means (±SD) of 25-35

2.2. Temporal trends

Figure 6 shows metal concentrations in mussels in the WS covering the period from 1979 – 2002. Generally, there were high fluctuations in metal concentrations between years. Generally, there were decreasing trends from 1979 to mid 1990s. However, in recent years, metal concentrations in mussels increased in some cases to levels similar to the 70s. For example, during the winter of 2000, Cd concentration of about 35 µg/g was recorded in mussels comparable to 38 µg/g in 1980 in the upper part of the WS estuary. However, in the lower estuary, such increases were less pronounced and in some cases (notably Cd and Ni) metals even appeared to decrease. In the case of copper, concentrations in mussels fluctuated up and down over the same period in both lower and upper estuary.

Results of a 2-way ANOVA testing the effects of *year* and *site* on metal concentrations for the period 1996, 1999 and 2002 showed a significant effect of both *site* and *year*, confirming that both factors had an effect on metal concentrations. In terms of *site*, metals were higher in the less saline sites (Hansweert and Ellewoutsdijk) than the more marine sites (Vlissingen and Westkapelle). Results also showed a significant interaction term between the two factors, implying that metal concentrations at the different sites varied differently during the study period. From Figure 6, it is clear that changes in the average metal concentrations between any two sampling times (years) are not always the same and sometimes not even in the same direction (i.e. Co, Mn).

2.3. Seasonal variations

Results of a 2-way ANOVA performed to investigate the effects of *season* and *site* (WS versus ES) on tissue metal concentrations. Both *season* and *site* had significant effects for all metals studied. The ANOVA results are consistent with trends observed in Figure 3 showing relatively higher tissue metal concentrations during the colder months with a peak around March-April, coinciding with the spawning period. When comparing sites, WS was consistently higher ($p<0.001$) than the less polluted ES. The biggest differences were observed for Cd which were always over 10-fold higher during all the sampling times. With respect to the interaction between site and season, with the exception of Cu, significant interactions between the two factors were observed, a further confirmation that though there were seasonal effects in both WS and ES, changes in the WS were much higher than in the ES.

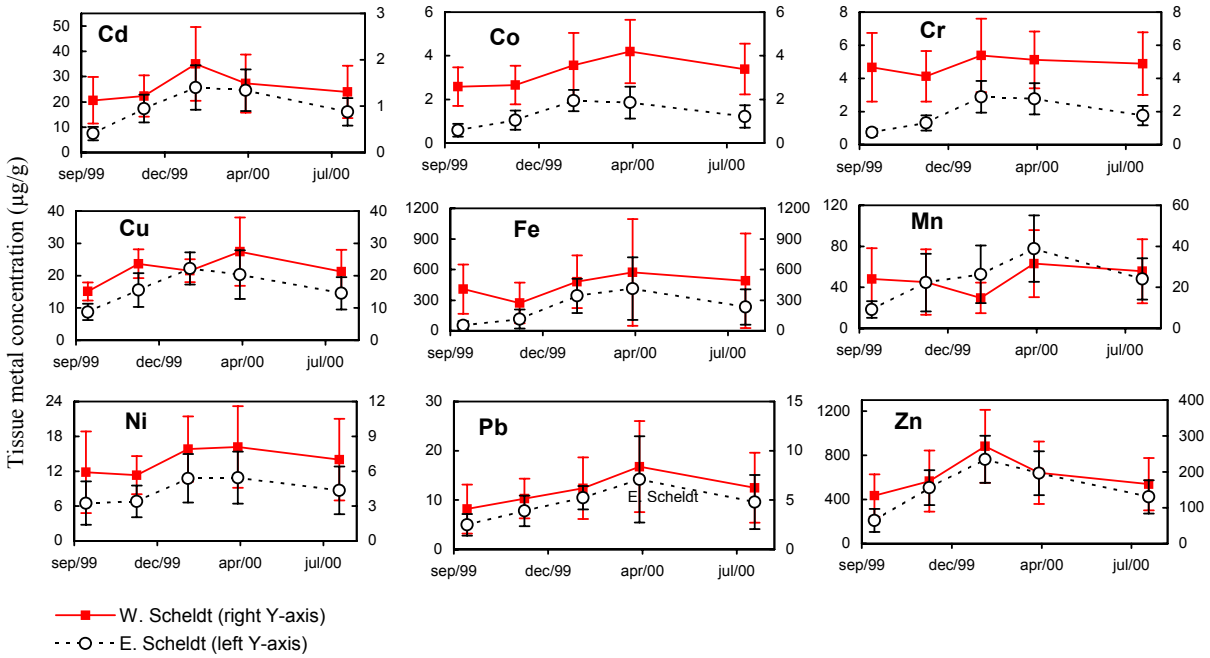


Figure 7. Seasonal variations in heavy metal concentrations (µg/g) in mussels (*M. edulis*) from Hansweert (WS) and Wemeldinge (ES) during the 1999 - 2000 sampling campaigns. The error bars are standard deviations of 25-35 individuals

2.4. Interpretation

Since the signing of international agreements to reduce toxic pollutants (Oslo, 1972 and Paris; 1974), Belgium and the Netherlands have recorded significant reductions in the discharges of heavy metals towards the North Sea, with values as high as 90% reductions (i.e. Cd, Cr) from 1985 to 1999. During the same period, specific efforts to restore the water quality parameters in the WS were beginning to show results (Nolting et al., 1999; Zwolsman & van Eck, 1999). As a result of these efforts, heavy metal concentrations in sediments, suspended particles and water in the WS have been on the decrease in the past 20 years (Regnier & Wollast, 1993; Zwolsman, et al., 1997; Baeyens, 1998; Zwolsman & van Eck, 1999; Nolting et al., 1999). However, this study has shown little evidence of decreasing trends regarding tissue metal concentrations in mussels from the WS in recent years. However, according to JMP data (1978-1992), there were big initial drops in metal concentrations soon after the implementation of pollution reduction programs in the early 80s (Luten et al., 1986; Stronkhorst, 1992). Unfortunately, the JMP monitoring exercise conducted by RIVO is restricted to only one or two sites in the WS estuary, though other sites around the Dutch coastal areas are included, and only for a few selected metals (Cd, Cu, Hg, Pb and Zn).

With respect to spatial variations, the current and earlier studies (Luten et al, 1986; Stronkhorst, 1992), all observed decreasing metal concentrations towards the North Sea. Increasing dilution of the metals with more saline water towards the sea and the

effect of removal processes such as sedimentation and complexation with increasing dissolved salts are probably the main causes of lower metal concentrations in mussels towards the sea. Recent studies have shown a clear trend of decreasing dissolved heavy metals in the WS towards the sea (Gerringa et al., 1996; Gerringa et al., 1998; Paucot & Wollast, 1997; Baeyens 1998; Baeyens et al., 1998a; Baeyens et al., 1998b; Zwolsman & van Eck, 1999). Another factor is the salinity gradient, laboratory experiments have shown results indicating that metal bioavailability decreases with increasing salinity, according to the concentrations of free metal ion species in the exposure water (Phillips, 1977; Blust et al., 1992; Bjerregaard & Depledge, 1994; Bervoets et al., 1995; Lee et al., 1998; Wang & Dei, 1999; Blackmore & Wang, 2003). When the ES and WS were compared, generally higher metal concentrations were recorded in the more polluted WS, sometimes by an order of magnitude (notably Cd). However, in the case of some essential metals (Cu, Fe, Mn and Zn), the differences were less, probably because as biological essential metals, concentrations of these metals in mussels are regulated up to a certain point.
The effect of season on metal concentrations in mussels showed similar seasonal patterns between the WS and the less polluted ES, with high values recorded during the winter and spring periods. However, there were also some interaction between site and season (2-way ANOVA) meaning that though the patterns were similar, the strength of the influence of season was relatively different in the two systems. A good example is demonstrated by Cd which showed bigger difference between spring and summer values in the WS compared to the ES. Such observations can be attributed to differences in seasonal changes in metal exposure between a polluted and less polluted environment. However, based on the fact that seasonal trends in the more polluted WS are very similar to the ES, suggests that seasonal changes in metal concentrations in mussels is probably more a result of changes in animal physiology than changes in metal exposure conditions. Mussels are known to show strong seasonal changes in physiological energetics, growth, reproduction and other related biochemical changes (Hawkins & Bayne, 1992). Any significant changes in these biological parameters may be the major causes of large seasonal variations in metal concentrations in wild mussel populations. Some studies have shown that changes in animal body weight due to seasonal changes in food availability, temperature or reproduction, often correlates with changes in metal concentrations (µg/g tissue weight), while metal body –burden (µg/ individual) remains unchanged (Philip, 1977; Cossa et al., 1979; Simpson, 1979; Wang & Dei, 1999; Wang & Fisher, 1997).

The influence of seasonal changes in metal exposure on metal concentrations in mussels can also be explained in terms of changes in river flow (i.e. small flow in winter resulting in less dilution of pollutants) and changes in the geochemistry of dissolved metals. In the Gironde estuary (southwest France) seasonal variations for arsenic and copper in mussels were strongly correlated with the river flow. In relation

to chemical speciation, studies have shown that the distribution of heavy metals is strongly influenced by salinity, dissolved organic carbon and dissolved oxygen in the WS (i.e. van Eck & De Rooij, 1993; Gerringa et al., 1998; Paucot & Wollast, 1997; Zwolsman et al., 1997). In spring when the freshwater inflow in the estuary is largely anoxic, dissolved metal concentrations tend to be very low especially in the upper estuary and metal partitioning in those conditions favours adsorption to suspended particles and sediments.

In terms of recent temporal trends, particularly in the upper part of the WS, Cd, Co, Cr, Ni and Zn were higher in 1999 and 2002 compared to 1996. However, mean concentrations of Cu and to a lesser extend Fe and Mn did not vary significantly over the same period. Lead was the only metal which appeared to decrease over the same period except in 1998 in the lowest salinity zone. With Cu, Fe and Mn showing similar patterns, that could suggest a distinction between essential and non-essential heavy metals. However, Zn another essential element was increasing. Mussels like most species are known to regulate the concentrations of essential metals. For this reason essential metals are not the most appropriate to give a good indication of ambient pollution levels. Decreasing Pb concentrations in mussels may be a result of phasing out of leaded gasoline in Europe in the 90s.

Of special interest are the increases in concentration observed for Cd, Cr, Ni and Zn since 1996 and particularly in the upper estuary. This is despite the significant cuts in the discharges of these metals since the 80s and efforts by environmental managers to restore water quality in the estuary. As explained earlier, the drop in tissue metal concentrations in the early 80s can be attributed to the decrease in metal input in the estuary. However, there is little indication of further changes in the concentrations of dissolved Cd, Cr, Ni or Zn. Therefore, the recent increases in mussel metal concentrations observed in the upper estuary are probably related to changes in the physical and chemical speciation of the metals resulting in increased availability to the mussels. This part of the estuary estuary also happens to be around the zone of maximum mixing in the WS (Zwolsman et al., 1997; Baeyens, 1998). Some physio-chemical processes including re-suspension of the bottom sediments are said to be the major factors controlling the metal composition of the particles in this part of the estuary (Baeyens et al., 1998a; 1998b; Gerringa et al., 1998; Nolting et al., 1999). In addition, redox conditions are also suggested to play an important role in releasing metals from the bottom sediments. Generally, in the WS, increased dissolved oxygen levels lead to oxidation of the bottom sediments which when re-suspended brings metals into the water column, causing secondary pollution (Zwolsman, et al., 1997; Paucot & Wollast, 1997; Baeyens et al., 1998b; Nolting et al., 1999).

Improved water quality conditions especially in the upper estuary in recent years and resulting remobilisation of metals from bottom sediments puts more metals in the

water column. Nolting et al., (1999) and Paucot and Wollast (1997) have showed variations in the ratio relating concentration of metals in suspended particles and in the dissolved phase (K_d) during the period 1978–1997. The variations were attributed to changing redox conditions in the estuary. Metal partitioning between particles and dissolved phases can be important for filter feeders such as mussels. Wang et al., (1996) showed that in mussels, the relative contribution to total metal body burden from ingested particles increases directly with increasing partitioning coefficient between particles and dissolved metals.

Other factors that may have influenced metal uptake by mussels in the upper WS estuary are changes in dissolved organic carbon (DOC) which changed significantly during the study period. Other parameters like suspended organic matter and chlorophyl-*a* did not seem to show strong temporal variations during the study period (WaterBase, www.waterbase.nl). In the early 80s, there were significant decreases in DOC in the whole estuary, but in recent years DOC again started to increase in the upper part of the estuary. Incidentally, these changes in DOC appear to coincide with observed trends in metal concentrations in mussels. It is generally known that by forming soluble complexes with most metals, DOC often tends to increase the concentration of dissolved metal fractions in the water. However, according to the chemical speciation modeling or the free ion activity model (FIAM), metal uptake in mussels is determined by the concentrations of free metal ion species. Therefore, according to FIAM, DOC-bound metals are expected to be less biologically available than other fractions. However, recent studies Kozuch & Pempkowiak (1996) showed a strong indication that marine humic substances enhanced the uptake of cadmium and copper in mussels, respectively. Other studies involving different aquatic species have also shown results which suggest that metal accumulation can be increased in the presence of dissolved organic carbon (Penttinen et al., 1995; Stuijfzand et al., 1999; Guo et al., 2001; Winch et al., 2002).

In conclusion, despite the improved water quality conditions in the Scheldt estuary, an increased accumulation of metals in mussels is observed especially in the upper estuary in recent years. This may be explained in terms of changes in physical and chemical speciation leading to increased bioavailability rather than new pollution loads into the estuary. However, the changes in metal speciation which may have caused these effects are currently difficult to re-construct due to lack of sufficiently detailed metal speciation data from the estuary (Gerringa et al., 1995). Zwolsman et al., (1997) predicted that the restoration of dissolved oxygen concentrations in the WS, which was the major goal of the management program implemented during the 70s and 80s, would lead to an increase in dissolved metal concentrations towards the North Sea. Perhaps, the findings of this study are a fulfilment of those predictions. Further, metal concentrations in mussels in the lower estuary did not appear to have increased significantly in recent times, indication of either a time-lag

effect or that the phenomenon is only restricted to the upper estuary which was largely anoxic during the peak of metal contamination in the 50s till the 70s (Nolting et al, 1999). For the better management of the estuary and as an example to similar estuaries around the world, detailed metal speciation studies should always be considered alongside biomonitoring programs, in order to correctly assess the potential risk of metal pollution in aquatic ecosystems.

3. Sediment-associated microbial communities

3.1. Bioavailable metals and organic matter.

The concentrations of bioavailable metals observed in the sediments from each sampling station are shown in Table 17. Except for Pb (similar Pb concentrations were observed in S1 and S2) the site S1 was always significantly most contaminated than S2. The site S4 was the least contaminated. The site S3 was very similar to S4 except for the bioavailable Zn (significantly higher in S3). The values of the contamination index (CI) decreased regularly from S1 to S4. These results confirm the presence of a heavy-metal gradient. The organic matter (OM) content of the sediment decreased from S1 to S4. Stations S3 and S4 did not differ significantly for the CI and the percentage of OM.

Table 17. Bioavailable metals (0.5 M HCl treatment), % of organic matter (%OM), and contamination index (CI) of the Sørfjord sediments. For metals, values are mean concentrations (μg/g of DW) ± SD (n=6); for OM, values are percentages of DW ± SD (n=3). Stations that share the same letter in a column (a-d) did not differ significantly (Tukey's test, $\alpha = 0.05$).

	Cd	Cu	Pb	Zn	**% OM**	**CI**
S1	1.18 ± 0.11 (a)	17.99 ± 3.80 (a)	112.74 ± 22.74 (a)	100.60 ± 7.44 (a)	3.8 ± 0.1 (a)	23.5 ± 3.6 (a)
S2	0.22 ± 0.02 (b)	8.17 ± 1.43 (b)	115.64 ± 10.10 (a)	68.54 ± 4.41 (b)	1.9 ± 0.01 (b)	14.7 ± 1.2 (b)
S3	0.25 ± 0.03 (b)	3.69 ± 1.78 (c)	13.98 ± 1.57 (b)	28.31 ± 3.33 (c)	1.1 ± 0.03 (c)	4.6 ± 0.8 (c)
S4	0.24 ± 0.08 (b)	2.87 ± 1.20 (c)	15.27 ± 2.84 (b)	20.46 ± 3.81 (d)	1.0 ± 0.04 (c)	4.0 ± 1.1 (c)

3.2. Microbial communities

(Complete results may be found in Gillan et al. 2005)

3.2.1. Direct counts

The number of cells observed in the sediment were 4.3 ± 0.7 (S1), 5.3 ± 1.1 (S2), 7.6 ± 0.9 (S3), and 13.4 ± 3.6 (S4) x 10^8 cells / g of sediments (DW). There were no significant differences between S1, S2, and S3 for the direct counts (Tukey test, P > 0.05). The cell density observed in S4 differred significantly from the cell densities observed at the other sites. These results show that the density of cells is not affected by extreme heavy-metal contaminations.

3.2.2. Cd resistance.

Results of the plate counts indicate that Cd resistance decreased from S1 to S4 (Fig.8). In S1, 100% of the colony forming units (CFU) were resistant up to 100 mg/l of Cd whereas in S4 only a few percent of the CFU were resistant to 50 mg/l. Cd resistance is thus higher, as expected, in Cd-polluted environments.

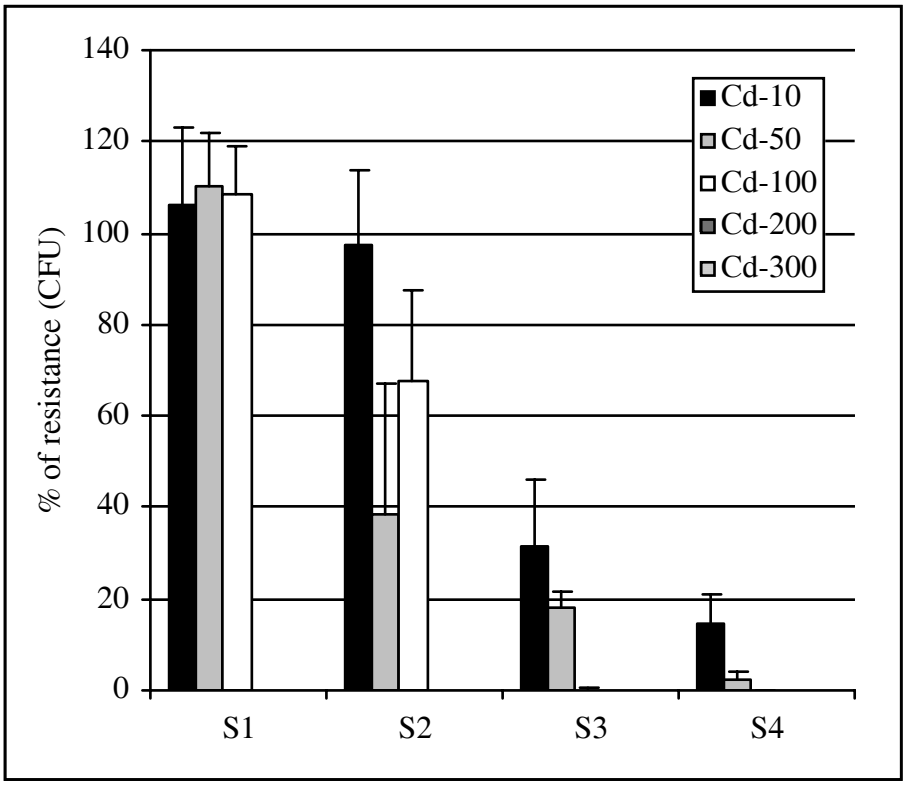


Figure 8. Percentage of Cd-resistant cells at the four sampling stations of the Sørfjord (n = 3). Agar plates contained 10 to 300 mg/l of $CdCl_2$.

3.2.3. DGGE analysis

A DGGE pattern is a fingerprint of the bacterial diversity. A Shannon index (H) of bacterial diversity may then be calculated using DGGE band positions and band intensities (Gillan 2004). The mean Shannon indexes were calculated for each station. H values were significantly higher in the most polluted sites S1 and S2 than

in the control site S4. This result indicate that extreme heavy-metal pollutions do not affect bacterial diversity in the marine environment or that bacterial diversity is able to recover.

3.2.4. Phylogenetic analysis

Four 16S rRNA clone libraries were constructed (one for each sampling station) and clones were partly sequenced with the primer 518F. After discarding sequences that were suspected of being chimaeric, diatom deriving sequences, and sequences that were of poor quality, 67 sequences of about 620 nucleotides remained (20 for S1; 21 for S2; 6 for S3; 20 for S4). The most numerous sequences belonged to three eubacterial groups : the γ-Proteobacteria (17 sequences), the δ-Proteobacteria (17 sequences), and the CFB bacteria (18 sequences). Some sequences grouped with the Planctomycetes, Actinobacteria, the Nitrospira group, and the α and β-Proteobacteria. A large proportion of the Sørfjord sequences (65%) was tightly associated with other clones found in cold marine sediments (shallow, continental shelf, or deep-sea sediments) such as those from the Antarctic, the Arctic, the Pacific, the Atlantic and the Black Sea.

3.2.5. FISH

Results of *in situ* hybridization are presented in Figures 9 and 10. It can be seen that the most contaminated station S1 presented the lowest percentage of eubacterial cells (4.9% of DAPI counts) in comparison to the other stations where values reached 65% of the DAPI counts. Archaebacteria were detected only in station S3 where they represented 1.4% of the DAPI counts. It may be concluded that heavy-metals have an influence on the type of bacterial group present. Planctomycetes represented 0.2% of the DAPI-stained cells in S1, and 6% in S3. The situation is similar for γ-Proteobacteria and δ-Proteobacteria.

3.2.6. Data correlations

A Pearson correlation matrix was calculated for bioavailable metals, DAPI counts, plate counts (Cd), organic matter and the signal of the FISH probes (Table 18). As expected, most bioavailable metals were positively and correlated to organic matter levels. The two bacterial groups that could be correlated to bioavailable metal levels were the γ-Proteobacteria (significant negative correlations for all metals) and the δ-Proteobacteria of the *Desulfosarcina-Desulfococcus* group (significant negative correlation only for Zn). Some DGGE bands (7 bands) had a relative intensy which was significantly and positively correlated to bioavailable metals.

3.2.7. Discussion

As shown by DGGE analysis, the bacterial diversities in S1 and S2 were comparable to the bacterial diversity of the un- contaminated site, S4. It may be suggested that the diversity of microbial communities in the Sørfjord were initially affected by the increasing levels of heavy metals, as was found in other microbial communities (Rasmussen et al 2001), but that after 80 years of heavy-metal contamination, the microbial communities have become as diverse as those living in uncontaminated places, such as S4. Previous workers, addressing Hg contamination of soils, suggested that recovery of lost genetic diversity is fast and begins immediately after contamination (Rasmussen et al 2001). As suggested here by DGGE analysis, each sampling station now has its own type of microbial community. Apparently, the recovery of the genetic diversity in S1 and S2 was not due to a reversion to the preexposure community but mainly to the appearance of new dominant species. Microbial diversity is thus a bad indicator of long-term heavy-metal pollution. Similar findings have been described in a heavy-metal-contaminated soil with a long his- tory of contamination (Ellis et al 2003). Good correlations between metals and organic matter were observed in the Sørfjord. Although such correlations are not proof of the complexation of the metals by the organic matter, it has been observed many times in various aquatic environments (Caccia et al 2003). The high levels of organic matter in S1 may be explained by high inputs due to the proximity of the city of Odda or to a high sedimentation rate. It might also be ex- plained by an inhibition of the activity of microorganisms due to the toxic effects of heavy metals. However, as biomass (i.e., DAPI counts) is not reduced in S1, it is more probable that S1 microorganisms are well adapted to grow in this contaminated environment. This is supported by other studies, which have shown that metal stress does not always reduce productivity or biomass (e.g. Shi et al 2002). We may provisionally conclude, until suitable productivity measurements are done, that cell density, and possibly productivity, is able to recover in marine sedi- ments contaminated by heavy metals.eubacteria FISH analysis revealed a clearly increasing abundance of □δ-Proteobacteria, □γ-Proteobacteria, and CFB bacteria from station S2 to station S4. These microbiological gradients might be the result of the metal gradient or of other environmental gradients. Metal effects are supported by highly significant correlation values (only for □γ-Proteobacteria and CFB bacteria). A previous report from the freshwater environment suggested that □γ-Proteobacteria were positively correlated to total metal levels (Feris et al 2003). However, in the present study, □γ-Proteobac- teria and CFB bacteria were negatively correlated to HCl- extractable metals (except Cd). This discrepancy can be ex- plained by differences in the microbial composition between freshwater and marine proteobacterial communities. Never- theless, two observations suggested that species positively cor- related to metals might be present in the Sørfjord: (i) as dis- cussed above, some sequences grouped with

clones from other heavy-metal-polluted sediments; (ii) some DGGE bands were detected in S1-S2 and not in S3-S4.

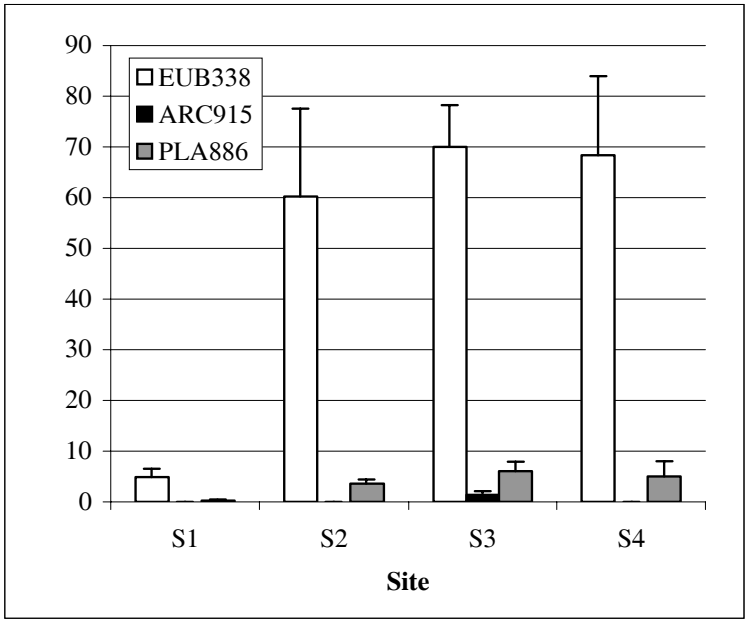


Figure 9. Community structure of the Sørfjord sediment-associated microbial communities as determined by fluorescent *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. Data are given as the percentage of DAPI-stained cells (n = 3).

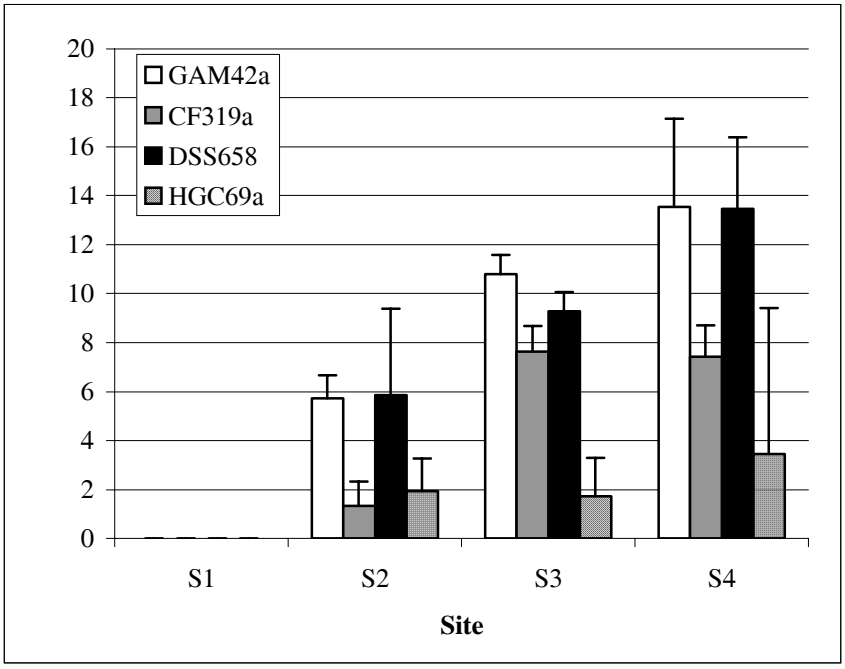


Figure 10. Community structure of the Sørfjord sediment-associated microbial communities as determined by fluorescent *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. Data is given as the percentage of DAPI-stained cells (n = 3).

Table 18.Correlations between bioavailable metals and microbial characteristics (Significant correlations in **bold**; $p < 0.05$)

	Cd	Cu	Pb	Zn
DAPI counts	- 0.463	- 0.543	- 0.686	- 0.716
DSS658	- 0.754	- 0.795	- 0.791	**- 0.889**
GAM42a	- 0.817	**- 0.876**	**- 0.865**	**- 0.960**
PLA886	- 0.694	- 0.733	- 0.665	- 0.761
OM	**0.939**	**0.943**	0.805	**0.952**
Cd-10	0.582	0.700	**0.905**	**0.905**
Cd-50	0.897	**0.918**	0.739	**0.888**

CONCLUSIONS AND RECOMMENDATIONS

In echinoderms, postmetamorphic stages appear rather resistant to the studied contaminations and, although effects at the individual level have been evidenced, no effect at the population level were revealed. Effects at this level are often difficult to establish due to numerous confounding natural stressors, like food, emersion, salinity etc. On the contrary, embryo production and larval survival are clearly at risk with some population producing no viable larvae on some year. This deleterious effect is probably compensated by the large dispersal capacity of the studied species. It appears that taking into account effects at the individual level for risk assessment policies is relevant and should be on the safe side when considering possible effects at the population level.

Data obtained on mussels in this project do not allow for now to answer the objective and it is not clear which population effects contaminants may have on these organisms.

The preliminary analysis of sediment-associated bacterial communities (SAMC) showed that these communities respond to metals and are possibly able to adapt to high contamination levels. Knowing the importance of these communities in metal speciation in the sediment, it seems very important to further study the interaction between metals and SAMC in order to understand how the latter are modified by metals and how they can affect metal bioavailability.

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