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Ecotoxicologie marine: distribution, flux, et effets biologiques de polluants métalliques chez l'astérie *Asterias rubens*, une espèce clef des biotopes littoraux de la Mer du Nord

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Résumé

Les facteurs gouvernant les flux de métaux lourds en milieu marin font des sédiments côtiers les cibles privilégiées des métaux lourds, contaminants préoccupants en Mer du Nord. De par leur position à l'interface eau-sédiments, les organismes benthiques sont particulièrement exposés aux métaux lourds. Parmi ces organismes, l'astérie *Asterias rubens* occupe de ce point de vue une position stratégique: en effet, elle se nourrit de divers invertébrés benthiques, dont des bivalves - bioaccumulateurs notoires de métaux lourds - et son statut d'espèce-clef fait que les effets des contaminants métalliques sur sa biologie pourraient avoir des répercutions sur l'ensemble de la communauté benthique. Le présent travail s'est attaché à définir les facteurs qui influencent les concentrations en métaux lourds chez *A. rubens*, à décrypter leurs flux au sein de l'espèce et à estimer leurs effets sur la biologie de l'astérie.

Les pourcentages de variation totale des concentrations métalliques chez les astéries adultes en relation avec différents facteurs (compartiment corporel, saison, station) ont été déterminés sur base d'analyses multifactorielles. Ces facteurs expliquent une grande partie (59 à 93%) de la variabilité des concentrations en Zn, Pb, Cd, Fe, Cr, Cu et Ti et 26% de la variabilité des concentrations en Hg. Le facteur "compartiment corporel" explique une grande partie de la variabilité des concentrations en métaux lourds (9,7 à 88% de la variabilité totale) en conditions non contaminantes. Les éléments de classes Ib et IIb (Zn, Cu, Cd, Hg) et le Fe sont principalement concentrés dans les caecums pyloriques, les autres éléments étudiés étant préférentiellement accumulés dans le squelette. La plupart des facteurs de bioconcentration par rapport aux proies sont inférieurs à 1 chez les astéries se nourrissant d'organismes filtreurs (ascidies et moules), ce qui indique que les métaux lourds ne sont généralement pas bioamplifiés dans de telles chaînes trophiques. Au contraire, la majorité des facteurs de bioconcentration chez les astéries se nourrissant d'organismes brouteurs (gastéropodes) sont supérieurs à 1. Des relations allométriques significatives ont été établies pour plusieurs métaux; les modèles descriptifs sont des relations de puissance positive (Cd dans le tégument et le système digestif) ou négative (Ti et Pb dans le système digestif), hyperbolique positive (Cr dans le tégument) ou linéaire positive (Zn et Cd dans le système digestif).

L'accumulation et l'élimination du Cd ont été modélisées lors de contaminations expérimentales *in situ*. Le Cd associé à la nourriture (moules précédemment exposées à 20 μg Cd l^{-1} pendant 2 mois et contenant 109 ± 29 μg Cd g^{-1} PS) ou présent dans l'eau de mer (5 ou 20 μg Cd l^{-1}) est accumulé dans les différents compartiments corporels. Dans les caecums pyloriques, un état d'équilibre est atteint après 20 jours de contamination, la concentration à l'équilibre variant de 3 à 9 μg Cd g^{-1} PS selon le mode d'exposition (*via* l'eau ou la nourriture). Ces concentrations à l'équilibre sont proportionnelles aux concentrations dans l'environnement dans la gamme 0,025 à

2,5 µg Cd l^{-1}. Le tégument et le squelette accumulent le Cd linéairement pendant toute la période d'exposition (45 jours). Les cinétiques d'élimination peuvent être décrites par des fonctions exponentielles inverses; elles sont significatives dans tous les compartiments sauf dans le squelette. Ces données promeuvent l'utilisation des caecums pyloriques comme bioindicateur à court-terme (de l'ordre de la semaine) de contamination par le Cd et celle du squelette comme bioindicateur à long-terme (de l'ordre du trimestre). La distribution et le devenir subcellulaire du Cd ont été étudiés dans les caecums pyloriques, organes cibles du Cd. Dans ces organes, la plus grande partie du Cd cytosolique se lie à des protéines de faibles masses moléculaires qui présentent plusieurs caractéristiques des métallothionéines (MTs): masse moléculaire (13 - 22 kDa), thermostabilité (90°C, 10 min), haut contenu en Cd et en groupe thiols (détermination par polarographie différentielle). Ces protéines sont saturées en Cd à l'issue des 10-20 premiers jours d'une contamination à 20 µg Cd l^{-1}. Leur quantification par analyse polarographique a permis de définir les niveaux de base en MTs (2,5 - 4,5 mg MT g^{-1} PS) dans des sites non contaminés de la Mer du Nord. Les concentrations en MTs d'astéries exposées au Cd (20 µg Cd l^{-1}) doublent après 30 jours. Le catabolisme des MTs est rapide et pourrait expliquer la rapide élimination du Cd des caecums pyloriques. Les astéries de certains sites fortement contaminés (Sørfjord, Norvège) présentent des concentrations en MTs (5 - 5,6 mg MT g^{-1} PS) significativement plus élevées que les niveaux basaux. Cependant, l'absence de relation simple entre la concentration en MTs et la biodisponibilité des MTs dans le fjord ne permet pas d'utiliser la quantification des MTs chez *A. rubens* comme biomarqueur de contamination.

L'accumulation et l'élimination du Pb ont été modélisées lors de contaminations expérimentales en aquarium. Lorsque les astéries sont exposées à 5, 20 ou 50 µg Pb l^{-1}, les cinétiques d'accumulation par les différents compartiments corporels sont décrites par des modèles de régression linéaire; aucune saturation n'est atteinte pendant les 30 jours d'exposition. A une concentration donnée, la vitesse de bioaccumulation est toujours significativement plus élevée dans le système digestif central que dans tout autre compartiment. Les constituants du système digestif éliminent le Pb selon une relation exponentielle inverse, les concentrations diminuant jusqu'aux valeurs initiales atteintes après 4 à 8 jours de dépuration. Le tégument élimine le Pb selon une relation linéaire. Par contre, les concentrations dans le squelette ne varient pas significativement pendant la période étudiée. Dans les gonades, les concentrations en Pb continuent à augmenter alors que la source exogène a disparu. Le Pb accumulé dans les gonades proviendrait du système digestif et serait éliminé lors de la ponte. Ce transfert de Pb depuis le système digestif serait effectif dès l'acquisition de la maturité sexuelle comme le suggère la relation allométrique observée pour ce dernier compartiment. Les cinétiques d'accumulation du Pb sur le squelette isolé de *A. rubens* ont été décrites lors de contaminations expérimentales (5, 20, 50 µg Pb l^{-1}) pendant 72 h. Deux étapes principales des cinétiques peuvent être distinguées. L'étape initiale rapide (30 premières minutes) est décrite par un modèle linéaire alors que l'étape suivante est décrite par un modèle à saturation, saturation qui est atteinte à l'issue des premières

24 heures. Ce modèle d'accumulation indique que le Pb est adsorbé sur la calcite magnésienne qui forme le squelette, ce phénomène jouant probablement un rôle important dans la bioaccumulation du Pb *in vivo*.

En se basant sur les paramètres cinétiques d'accumulation et d'élimination du Pb, le système digestif et le squelette de *A. rubens* peuvent être proposés comme bioindicateurs temporels complémentaires de contamination par le Pb, le système digestif indiquant une contamination récente (ordre de la semaine), le squelette intégrant les variations environnementales à plus longue échéance (ordre de la décennie). Ces bioindicateurs ont été validés dans un site fortement contaminé par les métaux lourds, dont le Pb (Sørfjord, Norvège). L'analyse des concentrations en Pb dans les caecums pyloriques et le squelette d'astéries prélevées le long du fjord a permis de confirmer la présence d'un gradient spatial de contamination par le Pb connu par ailleurs grâce à des analyses de sédiment: les concentrations en Pb dans les astéries collectées près de la source de contamination dépassaient les concentrations de base d'un facteur 4,4 dans les caecums pyloriques et 8,3 dans le squelette. Le gradient temporel de contamination du site (importante diminution de la biodisponibilité des métaux depuis 1992) a également été mis en évidence dans le squelette des astéries. Dans le site le plus contaminé, les concentrations en Pb dans le squelette des astéries de grande taille (12-14 cm de rayon, donc âgées de 4 à 7 ans) étaient 8 fois plus élevées que chez les individus les plus petits (0,5-4 cm, donc âgés de moins de 2 ans). Bien que l'accumulation du Pb dans le squelette soit probablement utilisée par *A.rubens* comme système de détoxication, il s'avère que cette accumulation n'est pas sans effet. En conditions expérimentales, la morphogenèse du squelette est altérée - quantitativement et qualitativement - chez les astéries exposées à des concentrations $\geq 5\ \mu g\ Pb\ l^{-1}$. *In situ* (Sørfjord), le développement squelettique des astéries des sites les plus contaminés est significativement altéré. De plus, ces astéries sont plus petites que les astéries des sites non contaminés.

Les résultats obtenus permettent de proposer *Asterias rubens* comme bioindicateur de contamination par le Cd et le Pb. Les organes à utiliser sont les caecums pyloriques (indicateur à court terme: ordre de la semaine) et le squelette (indicateur à long terme: ordre du trimestre pour le Cd, de la décennie pour le Pb). L'échantillonnage se portera sur des astéries de la plus grande classe de taille et sera effectué à la même période du cycle gamétogénétique dans les différentes populations étudiées. Des valeurs critiques au-delà desquelles toute concentration moyenne dans un échantillon sera considérée comme une indication de contamination sont proposées.

1. Introduction

Le contrat "Ecotoxicologie marine: distribution, flux, et effets biologiques de polluants métalliques chez l'astérie *Asterias rubens*, une espèce clef des biotopes littoraux de la Mer du Nord" s'est déroulé d'août 1992 à septembre 1996. Il a été entièrement réalisé par l'équipe du Laboratoire de Biologie marine sous la direction du Prof. M. Jangoux et du Dr. Ph. Dubois.

Les objectifs de ce contrat étaient:
(1) évaluer les niveaux de pollution métallique chez l'astérie *A. rubens* dans divers biotopes de la Mer du Nord,
(2) déterminer les flux de certains polluants métalliques chez cette espèce,
(3) estimer les effets biologiques de la pollution métallique chez *A. rubens*.

A l'issue de ce travail, les résultats obtenus permettent de considérer ces objectifs comme atteints (Rubrique 2: Résultats) et de proposer des applications (Rubrique 3: Applications scientifiques). Ces résultats ont fait l'objet de 11 articles scientifiques actuellement publiés, sous presse, soumis ou en préparation (Annexe I). Ils ont été présentés à l'occasion de 6 réunions scientifiques internationales (Annexe II). Une thèse de doctorat et deux mémoires de licence ont été réalisés dans le cadre du contrat (Annexe III). Les données pertinentes ont été déposées dans la base de données de l'Unité de Gestion du Modèle Mathématique de la Mer du Nord (UGMM).

L'ensemble des participants au contrat tiennent à exprimer leurs remerciements aux SSTC pour leur soutien financier et scientifique et à Monsieur J. Schoofs pour son aide logistique précieuse.

2. Résultats scientifiques

Les résultats scientifiques obtenus ont tous fait l'objet d'articles scientifiques actuellement publiés, sous presse ou soumis. Un rappel des résultats obtenus est proposé ci-après et les textes des articles sont repris *in extenso* en annexe du présent rapport (Annexe I).

2.1. Niveaux de contamination métallique chez *Asterias rubens*

La première partie de ce travail a constitué en un état des lieux de la contamination par les métaux lourds de populations de *Asterias rubens* vivant dans des zones éloignées des sources ponctuelles de contamination (ces données sont disponibles à l'UGMM sous la référence MUMM 94 CF.BE). On s'est plus particulièrement intéressé aux facteurs de variation des concentrations métalliques dans l'astérie. Les variations des concentrations en métaux lourds en fonction de la saison, de l'emplacement géographique et du compartiment corporel ont été déterminées chez des astéries prélevées dans quatre biotopes representatifs du littoral de la Mer du Nord à quatre périodes du cycle annuel gamétogénique. Les concentrations en Zn, Pb, Cd, Fe, Cr, Cu, Ti et Hg ont été mesurées dans les gonades, le tube digestif central, les caecums pyloriques, la paroi tégumentaire et le squelette. Selon les analyses multifactorielles de la variance, les facteurs considérés dans la présente étude comptent pour une grande partie (59 - 93%) de la variabilité totale des concentrations de tous les métaux étudiés sauf le Hg (2%). Le compartiment corporel apparaît être le facteur le plus important (jusqu'à 88% de la variabilité totale). Le facteur saisonnier apparaît secondaire (au maximum 8% de la variabilité totale). Cependant, pour un compartiment donné, les variations saisonnières peuvent présenter des rapports de 1,2 à 7,2 (15 pour le Ti). Les faibles interactions entre les facteurs "compartiment corporel" et "saison" indiquent que la bioaccumulation est synchrone au sein du corps des astéries. Les concentrations en Zn, Cd, Cu, Hg et Fe sont significativement plus élevées dans les caecums pyloriques. Les autres éléments (Pb, Cr, Ti) sont préférentiellement accumulés dans le squelette. La plupart des facteurs de bioconcentration (BCF) par rapport aux proies sont plus petits que 1 chez les astéries se nourrissant d'organismes filtreurs (ascidies et moules), ce qui suggère que la plupart des métaux lourds ne sont pas bioamplifiés. Au contraire, la majorité des facteurs de bioconcentration chez les astéries se nourrissant d'organismes brouteurs (gastéropodes) sont supérieurs à 1. La variabilité associée au facteur compartiment indique qu'il faut éviter d'utiliser *A. rubens* comme un ensemble homogène dans les programmes de biomonitoring. La variabilité saisonnière doit également être prise en compte.

Les variations de concentrations en métaux lourds en fonction de la taille (âge) ont été déterminées dans une population de l'Escaut oriental. Cent astéries représentatives de la gamme de taille (1 - 16 cm) de la population étudiée ont été prélevées, mesurées et disséquées. Les concentrations en Zn, Pb, Cd, Fe, Cr, Cu et Ti ont été mesurées dans le système digestif et le tégument. La distribution des fréquences de tailles mesurées dans la population a pu être décomposée en 4 cohortes, ce qui indique que ces astéries vivent au moins 4 ans. Les relations entre taille et concentrations ont été modélisées par régressions linéaires après transformations adéquates des données. Dans le compartiment "Tégument", les concentrations en Cd et en Cr augmentent avec la taille selon une fonction de puissance et une fonction hyperbolique, respectivement; les concentrations variant de 0,3 à 0,8 µg Cd g^{-1} PS et 0,5 à 3,3 µg Cr g^{-1} PS. Les concentrations des autres éléments étudiés ne varient pas significativement avec la taille des astéries. Dans le compartiment "système digestif", les concentrations en Cr (0,5 - 0,8 µg Cr g^{-1} PS) et en Zn (28 - 195 µg Zn g^{-1} PS) augmentent linéairement avec la taille alors que les concentrations en Cd (0,1 - 2,8 µg Cd g^{-1} PS) augmentent selon une fonction de puissance. Au contraire, les concentrations en Pb (3,8 - 0,3 µg Pb g^{-1} PS) et en Ti (2,6 - 0,02 µg Ti g^{-1} PS) diminuent avec la taille selon une fonction de puissance inverse. Les concentrations en Fe et en Cu ne varient pas significativement avec la taille. La diminution des concentrations en Pb correspond à une taille (4 - 5 cm) d'acquisition de la maturité sexuelle et de croissance somatique rapide. A ce stade, les flux de Pb depuis le système digestif vers les gonades et le squelette seraient accélérés, ce qui diminuerait les concentrations instantanées dans le système digestif. Les variations allométriques étant les plus faibles dans la plus grande classe de taille, c'est cette dernière qui devrait être échantillonnée dans les programmes de biomonitoring utilisant *A. rubens* comme espèce de référence.

2.2. Flux du Cd et du Pb chez *Asterias rubens*

Afin de comprendre le comportement des métaux lourds appartenant à chacune des deux catégories de métaux distinguées (métaux concentrés préférentiellement dans le système digestif et dans le tégument, respectivement), les flux dans *A. rubens* d'un métal de chaque catégorie ont été déterminés. Les métaux choisis (Cd et Pb) appartiennent au groupe des métaux préoccupants en Mer du Nord (NSTF 1993).

L'accumulation et l'élimination du Cd ont été modélisées lors de contaminations expérimentales *in situ*. Le Cd associé à la nourriture (moules précédemment exposées à 20 µg Cd l^{-1} pendant 2 mois et contenant 109 ± 29 µg Cd g^{-1} PS) ou présent dans l'eau de mer (5 ou 20 µg Cd l^{-1}) est accumulé dans les différents compartiments corporels (caecums pyloriques, tégument et squelette). Le Cd présent dans l'eau de mer (1 µg Cd l^{-1}) est également accumulé significativement dans le tégument mais pas dans les caecums pyloriques ou le squelette. Dans les caecums pyloriques, un état d'équilibre est atteint après 20 jours de contamination, la concentration à l'équilibre variant de 3 à 9 µg Cd g^{-1} PS selon le mode d'exposition (*via* l'eau ou la nourriture). Le tégument et le squelette accumulent le Cd linéairement pendant toute la période d'exposition (45 jours) à la vitesse de 0,07 à 0,25 µg Cd g^{-1} PS j^{-1} selon le mode d'exposition. Les cinétiques d'élimination peuvent être décrites par des fonctions exponentielles inverses; elles sont significatives dans tous les compartiments sauf dans le squelette. Les résultats montrent qu'il y a un flux de Cd à travers l'organisme depuis le système digestif vers la paroi tégumentaire au sein de laquelle le Cd est lentement incorporé dans le squelette. Les facteurs de concentration calculés pour les caecums pyloriques sont inclus dans la gamme de valeurs rapportées dans la littérature pour des expériences en laboratoire; la présente étude valide sur le terrain ces expériences. Il en résulte que les concentrations en Cd des caecums pyloriques d'*A. rubens* sont proportionnelles aux concentrations dans l'environnement dans la gamme 0,025 à 2,5 µg Cd l^{-1}. L'élimination étant relativement rapide, les caecums pyloriques d'*A. rubens* peuvent donc être considérés comme un bioindicateur d'une contamination récente par le Cd (ordre de la semaine).

Les effets des contaminations par le Cd sur les concentrations en Cu et en Zn (deux métaux essentiels pris en charge par des métallothionéines) ont été déterminés. Tandis que les concentrations en Cu ne varient pas significativement, les concentrations en Zn augmentent brusquement (42 à 134%) dans les caecums pyloriques pendant les 30 premiers jours de contamination quel que soit le mode d'exposition. La contamination par le Cd déclencherait une entrée de Zn qui pourrait atténuer les effets délétères du Cd et déclencher la synthèse de métallothionéines.

L'accumulation et l'élimination du Pb ont été modélisées lors de contaminations expérimentales *via* l'eau en aquarium. Lorsque les astéries sont exposées à 5, 20 ou 50 µg Pb l^{-1}, les cinétiques d'accumulation par les différents compartiments corporels sont décrites par des modèles de régression linéaire; aucune saturation n'est atteinte à l'issue des 30 jours d'exposition. Dans tous les compartiments, la vitesse d'accumulation augmente significativement avec la concentration contaminante. A une concentration donnée, la vitesse de bioaccumulation est toujours significativement plus élevée dans le système digestif central que dans tout autre compartiment. Les constituants du système digestif éliminent le Pb selon une relation exponentielle inverse, les concentrations diminuant jusqu'aux valeurs initiales atteintes, après 4 à 8 jours de dépuration. Le tégument élimine le Pb selon une relation linéaire. Par contre les concentrations dans le squelette ne varient pas significativement pendant la période étudiée. Dans les gonades, les concentrations en Pb continuent à augmenter après que la source exogène ait disparu. Le Pb accumulé dans les gonades proviendrait du système digestif. Ce transfert de Pb depuis le système digestif serait effectif dès l'âge de maturité sexuelle comme le suggère la relation allométrique observée pour ce dernier compartiment. Le Pb accumulé dans les gonades serait ensuite expulsé lors de la ponte. Selon les paramètres d'accumulation et d'élimination, le tube digestif central et le squelette sont proposés comme bioindicateurs temporels complémentaires de contamination par le Pb, le système digestif indiquant une contamination récente (ordre de la semaine), le squelette intégrant les variations environnementales à plus longue échéance (plusieurs années).

Les cinétiques d'accumulation du Pb sur le squelette isolé ont été décrites lors de contaminations expérimentales (5, 20, 50 µg Pb l^{-1}) pendant 72 h. Deux étapes principales peuvent être distinguées dans ces cinétiques. Une étape initiale rapide (30 premières minutes) est décrite par un modèle linéaire, l'étape suivante est décrite par un modèle à saturation, saturation qui est atteinte à l'issue des premières 24 heures. La concentration à saturation dépend de la concentration en Pb dissous. La quantité de Pb accumulée par le squelette varie de 44 à 57 % du Pb total dissous. Une comparison avec les modèles d'accumulation établis pour d'autres métaux sur de la calcite pure suggère que le Pb accumulé par le squelette dans l'astérie est adsorbé, ce phénomène d'adsorption jouant probablement un rôle important dans la bioaccumulation du Pb *in vivo*.

2.3. Effets du Cd et du Pb chez *Asterias rubens*

Le Cd pénètre dans l'astérie par le tégument et la paroi digestive. L'accumulation dans les caecums pyloriques est rapide et peut être décrite par une courbe à saturation. Il n'y a quasi pas de transfert vers les gonades. L'incorporation du Cd dans le squelette est plus lente que celle du Pb. Il est probable que la majeure partie du Cd accumulé dans les caecums pyloriques soit prise en charge par des protéines de type métallothionéines. La quantification de celles-ci fournirait donc une mesure de la réponse physiologique de l'astérie face à la contamination par le Cd. Les effets du Cd peuvent donc être appréhendés à travers la quantification des métallothionéines dans les caecums pyloriques; ceci a été mené en parallèle avec la mesure de l'activité de la phosphatase alcaline, choisie comme marqueur de l'état cellulaire général.

La distribution et le devenir subcellulaire du Cd ont été étudiés dans les caecums pyloriques, organes cibles du Cd d'astéries contaminées expérimentallement ou "naturellement". La répartition du Cd entre les différents pools intracellulaires a été déterminée par spectrométrie d'absorption atomique avec four graphite des fractions cytosoliques traitées à la chaleur puis séparées par chromatographie sur gel. Les protéines de faibles masses moléculaires lient >80% du Cd cytosolique et sont saturées par le métal lors de contaminations expérimentales à 20 µg Cd l^{-1} pendant 10 à 20 jours. Ces protéines présentent plusieurs caractéristiques des métallothionéines (MTs): masse moléculaire (13 - 22 kDa), thermostabilité (90°C, 10 min), haut contenu en Cd et en groupe thiols (détermination par polarographie différentielle). L'analyse des fractions chromatographiques indique que la majorité des groupes thiols (66-73%) présents dans le cytosol traité thermiquement sont associés aux MTs. Des déterminations subséquentes des concentrations en MTs ont été faites en utilisant des extraits cytosoliques totaux et en tenant compte de l'interférence générée par les groupes thiols présents dans les protéines résiduelles, thermo-stables et de haute masse moléculaire. Les caecums pyloriques des astéries collectées dans des sites non pollués du SO de l'Angletterre, du SO des Pays-Bas et du SO de la Norvège contiennent des niveaux de base en MTs de 2,5 - 4,5 mg MT g^{-1} PS. Les concentrations en MTs des caecums pyloriques d'astéries exposées au Cd (20 µg Cd l^{-1}) doublent après 30 jours. Le catabolisme des MTs est rapide et pourrait expliquer la rapide élimination du Cd des caecums pyloriques. Les astéries de certains sites fortement contaminés (Sørfjord, Norvège) présentent des concentrations en MTs (5 - 5,6 mg MT g^{-1} PS) significativement plus élevées que les niveaux basaux. Cependant, l'absence de relation simple entre la concentration en MTs et la biodisponibilité des MTs dans le fjord ne permet pas d'utiliser la quantification de MTs chez *A. rubens* comme biomarqueur de contamination. L'activité de la phosphatase alcaline n'est pas affectée significativement lors de contaminations par le Cd en laboratoire.

Le Pb pénètre dans l'astérie principalement par la paroi digestive et est rapidement distribué dans l'organisme. Une partie est assimilée par les gonades pendant leur croissance mais la majeure partie est acheminée vers la paroi tégumentaire où elle est piégée dans le squelette. L'incorporation des métaux lourds dans le squelette peut être considérée comme un processus de détoxication par compartimentation. Il n'est cependant pas exclu que ces métaux affectent la squelettogenèse. Les effets de la bioaccumulation du Pb sur la morphogenèse du squelette ont été déterminés. La regénération des piquants qui bordent la gouttière ambulacraire (les piquants adambulacraires a été utilisée comme modèle expérimental. La regénération des piquants a été initiée chez des astéries exposées expérimentalement à 1, 5, 20 ou 50 µg Pb l^{-1} pendant 15 jours. La bioaccumulation du Pb dans la paroi tégumentaire, le squelette et les piquants adambulacraires est dose-dépendante. Une étude en microscopie électronique à balayage a montré que la morphogenèse du squelette est affectée: les trabécules sont plus courtes et plus trapues, les ponts transversaux secondaires ne sont pas formés après 15 jours, la forme des trabécules est irrégulière et le dépôt minéral est fortement réduit. La taille des regénérats est significativement plus petite chez les individus exposés aux concentrations $\geq$ 5 µg Pb l^{-1} (jusqu'à 70% de réduction). L'activité de la phosphatase alcaline a été mesurée comme marqueur de l'état général de la paroi tégumentaire; elle ne diminue significativement (30% de réduction) que chez les individus exposés à 50 µg Pb l^{-1}. Ces résultats indiquent que, par comparaison avec d'autres phylums, la squelettogenèse chez les astéries est particulièrement sensible au Pb et que les effets toxiques du Pb peuvent dépendre directement de son incorporation dans le squelette.

L'approche expérimentale des effets des métaux lourds sur *A. rubens* a été complétée par une approche sur le terrain afin d'établir l'impact d'une contamination réelle (et multiple, i.e. par plusieurs métaux simultanément). Pour ce faire, des astéries ont été collectées en août 1995 dans le Sørfjord (SO Norvège), un fjord dans lequel des résidus chargés de métaux lourds ont été

Tableau 1: Caractéristiques qui permettent d'envisager l'usage de *Asterias rubens* comme bioindicateur de contamination par les métaux lourds.

Qualités d'un bioindicateur idéal *	Caractéristiques d'*Asterias rubens*	Références
Ubiquité	Distribution du genre couvre l'hémisphère Nord, depuis le littoral jusqu'à -650 m, espèce euryhaline	Hayward & Ryland 1990
Stabilité des populations au cours de l'année	Espérance de vie: jusqu'à 7 ans	Guillou & Guillaumin 1985
Permanence de localisation	Comportement sédentaire	Hostens & Hammerlynck 1994
Abondance	Populations composées de milliers d'individus	Hostens & Hammerlynck 1994
Facilité d'échantillonnage	A la main à marée basse, par dragage, ou en scaphandre autonomome	
Taille suffisante pour analyses	Tégument, squelette, caecums pyloriques, et gonades des spécimens adultes pèsent plusieurs g PS	
Bonne connaissance de la biologie générale de l'organisme	Milliers de publications sur sa biologie depuis la fin du 19° siècle	
Etude facile	Acclimatation rapide aux conditions en laboratoire	
Forte affinité pour les métaux lourds	Démontrée pour Sr, Pu, Cd, Hg, Se, Mn, Pb	Binyon 1978, Guary *et al* 1982, Besten *et al* 1990, Sorensen & Bjerregaard 1991, Rouleau *et al* 1993, Hansen & Bjerregaard 1995, Présent travail
Concentration tissulaire détectable	Détection aisée des concentrations tissulaires par spectrométrie d'absorption ou d'émission	Besten *et al* 1990, Sorensen & Bjerregaard 1991, Everaarts & Fischer 1989, Présent travail
Sensibilité physiologique aux métaux lourds	Sensibilité au Cu, au Pb et au Cd	Gerets *et al* 1972, Besten *et al* 1989, Présent travail
Relation entre les concentrations tissulaires et les concentrations dans le milieu	Démontrée pour le Cd, le Pb et le Zn	Bjerregaard 1988, Présent travail
Présence en zone polluée	Présence dans le Sørfjord (SO Norvège), site le plus pollué par les métaux lourds en Mer du Nord	Présent travail

*: d'après Phillips (1976, 1990), Bryan & Hummerstone (1977) et Bryan (1984).

déversés depuis plus de 65 ans par des fonderies situées à son amont. Les déversements ont diminué regulièrement depuis 1986 et, en 1992, des actions supplémentaires ont été entreprises afin de réduire la biodisponibilité des métaux en couvrant d'une "membrane" les sédiments de la région fortement contaminée située à proximité de la fonderie principale. Les astéries appartenant à la plus grande classe de taille ont été collectées dans quatre sites choisis le long du fjord. De plus, des astéries de différentes tailles (âges) ont été collectées à l'amont du fjord. Les concentrations en Zn, Pb et Cd ont été mesurées dans les caecums pyloriques, la paroi tégumentaire et le squelette de ces astéries (ces données sont déposées à l'UGMM, référence non encore disponible). Le gradient géographique de contamination par les métaux le long du fjord se traduit, comme attendu, dans les compartiments corporels des astéries: les concentrations métalliques sont toujours plus grandes (1,7 à 8,3 fois) dans les astéries appartenant à la plus grande classe de taille échantillonnées à proximité de la fonderie principale. A cet égard, il est intéressant de noter que la moule *Mytilus edulis*, bioindicateur largement utilisé dans le monde, ne reflète pas ce gradient. Les différences indiquées par les caecums pyloriques (reconnus comme bioindicateur à court terme), et la paroi tégumentaire et le squelette (reconnus comme bioindicateurs à long terme) montrent que la biodisponibilité des métaux a diminué ces dernières années. Ceci est confirmé par l'étude des variations allométriques des concentrations métalliques dans la population du site le plus contaminé. Dans cette population, les concentrations en Pb dans le squelette des astéries appartenant à la plus grande classe de taille (12 - 14 cm; 154 ± 63 μg Pb g^{-1} PS) sont 8 fois plus grandes que celles mesurées dans le squelette des juveniles (0,5 - 4 cm; 19 ± 8 μg Pb g^{-1} PS). Les astéries de 12 - 14 cm sont vraisemblablement âgées de 4 - 7 ans (*i.e.* recrutées avant la pose de la "membrane") tandis que les petites astéries sont vraisemblablement nées plus tard (*i.e.* recrutées après la pose de la "membrane"). Une plus petite taille et une réduction du squelette ont été observées chez les astéries les plus âgées de cette population; cet effet serait dû à la contamination par les métaux lourds. Les concentrations métalliques élevées mesurées dans les compartiments corporels des astéries collectées en amont du fjord suggèrent que la biodisponibilité des métaux est encore importante dans ce site.

2.4. Discussion et conclusion: valeur bioindicatrice de *Asterias rubens* et sensibilité vis-à-vis des métaux lourds

A l'entame de ce travail, on savait que *A. rubens* répondait à plusieurs des caractéristiques requises d'un bon bioindicateur de contamination par les métaux lourds (Tableau 1), à savoir les caractéristiques qui pourraient être qualifiées d'écologiques ou de morphologiques. Le présent travail a permis de démontrer l'adéquation des caractéristiques écotoxicologiques de *A. rubens* (en ce qui concerne le Cd et le Pb). Il en résulte que (1) les caecums pyloriques de *A. rubens* peuvent être utilisés comme bioindicateur à court terme (ordre de la semaine) de contamination par le Cd et le Pb, (2) le squelette comme bioindicateur à moyen terme (ordre du trimestre) de contamination par le Cd et à long terme (ordre de la décennie) par le Pb. Ces compartiments devront être échantillonnés sur des astéries appartenant à la plus grande classe de taille et récoltées à la même saison.

En tenant compte de la relation taille - âge des astéries et du long temps de rétention du Pb dans le squelette, une étude allométrique de la concentration en Pb dans le squelette des astéries permet en outre de retracer l'histoire de la contamination du site, tel que cela a été réalisé dans le Sørfjord.

Les résultats engrangés lors de l'étude des variations naturelles des concentrations métalliques chez *A. rubens* fournissent une population de données représentant la gamme de fluctuation de ces concentrations en milieu non contaminé. Le percentile 95 de cette distribution peut être choisi (pour chaque métal) comme une valeur critique au-delà de laquelle toute concentration moyenne dans un échantillon sera considérée comme une indication de contamination. Vu les variations entre compartiments, il y a lieu de définir des valeurs critiques différentes pour chacun de ceux-ci. Ces valeurs sont reprises au Tableau 2 pour les compartiments retenus et pour les deux métaux dont on a pu montrer que les concentrations reflètent les conditions environnementales.

Tableau 2: Valeurs critiques (µg métal g^{-1} PS) des concentration en métaux lourds dans les compartiments corporels de *A. rubens* adultes au-delà desquelles une contamination doit être suspectée. CP: caecums pyloriques.

	Squelette	CP
Pb	10	1,1
Cd	1,2	5,1

La proposition d'une nouvelle espèce bioindicatrice de contamination par les métaux lourds peut sembler peu pragmatique face à l'emploi important des moules *Mytilus* spp. dans cette fonction. Le but n'est toutefois pas de remplacer les unes par l'autre mais bien de proposer leur usage conjoint, se ralliant ainsi à l'argument de Gray (1989) selon qui l'étude de plusieurs espèces est préférable afin d'indiquer l'état de contamination d'un écosystème, le bioindicateur universel n'existant pas. Ceci peut être illustré (aux dépens de *Mytilus*) par les travaux de Coleman *et al* (1986) qui ont montré qu'il n'existe pas de relation simple entre les concentrations corporelles en Cd chez *Mytilus edulis* et les concentrations dans l'eau. Similairement, les concentrations corporelles en Cd, Pb, Cu et Zn dans les moules du Sørfjord ne reflètent pas les gradients de contamination dans le fjord (NSTF 1993). Notons aussi que les moules ne sont pas ubiquistes [ex: distribution limitée à l'infralittoral, absence de certains biotopes - comme les herbiers de phanérogammes]. Finalement, l'utilisation de l'exosquelette des bivalves pour retracer l'histoire d'une contamination a été écartée par Phillips (1980). Dans cette optique, Phillips (1990) fait la synthèse des informations sur les bioindicateurs disponibles à l'époque, bioindicateurs qu'il regroupe en trois catégories: les algues macrophytes, les crustacés et les mollusques. Chacune de ces catégories présente des avantages et des inconvénients, ce qui peut être frustrant si l'on s'acharne à vouloir définir *le* bioindicateur idéal. La réponse serait donc de choisir des espèces présentant des informations complémentaires et ayant de préférence le statut d'espèce-clef. Pour être complémentaires, ces espèces devraient être choisies parmi des taxons biologiquement éloignés. Dans cette optique, *M. edulis* et *A. rubens* sont complémentaires. L'une est un protostomien filtreur pourvu d'un squelette externe qui maintien les autres tissus éloignés des sédiments [les moules se fixent sur les substrats durs mais peuvent aussi se fixer en agrégats sur les substrats meubles (Hayward & Ryland 1990)]; l'autre est un deutérostomien prédateur à squelette interne et dont certaines surfaces respiratoires - les pieds ambulacraires - sont en contact direct avec les sédiments. Il en résulte de ces différences biologiques que les deux espèces présentent des comportements très différents vis-à-vis des métaux lourds. L'étude conjointe de telles espèces serait vraisemblablement plus représentative de la biodiversité des écosystèmes marins. Les algues macrophytes complèteraient avantageusement ce tandem, ces organismes accumulant essentiellement les métaux lourds en phase dissoute (Phillips 1990).

A. rubens apparaît comme une espèce relativement résistante aux métaux lourds: c'est une des seules espèces de macro-invertébrés qui survit dans les sites les plus pollués du Sørfjord. (Cette résistance augmente d'ailleurs sa valeur bioindicatrice.) Des effets sublétaux ont cependant été mesurés à différents niveaux d'organisation biologique en conditions expérimentales et dans la nature. Au niveau subcellulaire, une inhibition significative de l'activité de la phosphatase alcaline a été mesurée dans les caecums pyloriques d'astéries prélevées dans les sites les plus contaminés du Sørfjord. Au niveau de l'organisme, une sensibilité tant qualitative que quantitative de la morphologie du squelette au Pb a été montrée. Ces effets du Pb sur la squelettogenèse ont été démontrés expérimentalement et rendent probablement également compte du faible développement du squelette des astéries des site les plus contaminés du Sørfjord. Au niveau de la population enfin, un effet des métaux sur la distribution de fréquence des tailles dans les mêmes sites du Sørfjord a pu être mis en évidence.

3. Applications scientifiques

Le présent travail a démontré que l'astérie commune *A. rubens* présente toutes les caractéristiques requises d'un bon bioindicateur de contamination par le Cd et le Pb. La sensibilité de cette espèce en tant que bioindicateur s'est révélée complémentaire voire supérieure à celle de *Mytilus edulis*. Il est dès lors proposé d'employer *A. rubens* de concert avec *M. edulis*. (La nécessité d'employer plusieurs bioindicateurs appartenant à des groupes aussi distincts que possible est maintenant admise de façon générale.)

Les organes à utiliser dans le cadre d'un programme de surveillance sont les caecums pyloriques (indicateur à court terme: ordre de la semaine) et le squelette (indicateur à long terme: ordre deu trimestre pour le cd, de la décennie pour le Pb). l'échantillonnage des différentes populations à étudier doit être effectué à la même période du cycle gamétogénique (quelle qu'elle soit) et doit concerner les individus de la plus grande classe de taille. Les valeurs critiques au-delà desquelles toute concentration moyenne dans un échantillon sera considéré comme une indication de contamination sont (µg métal g^{-1} PS):

	Squelette	CP
Pb	10	1,1
Cd	1,2	5,1

L'histoire (dernière décennie) de la contamination d'un site par le Pb pourra également être reconstituée en établissant les relations entre les concentrations en Pb dans le squelette et la taille des astéries. Pour ce faire il y a lieu d'échantillonner toutes les classes de taille de la population considérée.

Annexe I. Publications

- Quatre articles publiés:

(1) Temara A, Warnau M, Ledent G, Jangoux M, Dubois P (1993) Distribution anatomique des métaux lourds chez l'astérie *Asterias rubens* en période de pré-ponte du cycle gonadique. Résultats péliminaires. *In*: *Qualité du milieu marin - Indicateurs biologiques et physico-chimiques*. Boudouresque CF, Avon M, Pergent-Martnini C (eds) GIS Posidonie publ. Fr. pp. 135-139

(2) Temara A, Aboutboul P, Warnau M, Jangoux M, Dubois P (1995) Kinetics of lead uptake by the skeleton of the asteroid *Asterias rubens* (Echinodermata). *In: Echinoderm Research 1995*, Emson RH, Smith AB, Campbell AC (eds) Balkema, Rotterdam, pp. 79-82

(3) Temara A, Warnau M, Ledent G, Jangoux M, Dubois P (1996) Allometry of metal bioconcentration in the asteroid *Asterias rubens*. *Bull Environm Contam Toxicol* 56: 98-105

(4) Temara A, Ledent G, Warnau M, Paucot H, Jangoux M, Dubois P (1996) Experimental cadmium contamination of *Asterias rubens*, L. (Echinodermata). *Mar Ecol Prog Ser.* 140: 83-90

- Deux articles acceptés pour publication:

(5) Temara A, Warnau M, Dubois P, Langston WJ. (in press) Quantification of metallothioneins in the common asteroid *Asterias rubens* (Echinodermata) exposed experimentally or naturally to cadmium. *Aquatic Toxicol*

(6) Temara A, Aboutboul P, Warnau M, Jangoux M, Dubois P. Uptake and fate of lead in the common asteroid *Asterias rubens* (Echinodermata). *Water, Air and Soil Pollution*

- Quatre articles soumis:

(7) Temara A, Warnau M, Jangoux M, Dubois P. Factors controlling heavy metal concentrations in the asteroid *Asterias rubens* (Echinodermata).

(8) Temara A, Skei J, Gillan D, Warnau M, Jangoux M, Dubois P. Temporal and spatial trends of heavy metal contamination in the polluted Sørfjord (SW Norway).

(9) Temara A, Langston WJ, Warnau M, Jangoux M, Dubois P. *Asterias rubens* (Echinodermata, Asteroidea) in polluted Sørfjord (SW Norway): molecular biomarkers and bioavailability of heavy metals".

(10) Temara A, Warnau M, Jangoux M, Dubois P. Effects of exposure to cadmium on the concentrations of essential metals in *Asterias rubens* (Asteroidea).

Un article en préparation

(11) Temara A, Nguyen QA, Warnau M, Jangoux M, Dubois P. High sensitivity of skeletogenesis to Pb in the asteroid *Asterias rubens* (Echinodermata)

- Deux abstracts publiés

(1) Temara A, Warnau M, Ledent G, Jangoux M, Dubois P (1994) Heavy metals in the asteroid *Asterias rubens* and its prey *Mytilus edulis*: seasonal and geographical variations in four selected North Sea biotopes. *In: Echinoderms through time.* David B, Guille A, Féral JP, Roux M (eds), A.A. Balkema, Rotterdam, p. 367

(2) Temara A, Aboutboul P, Warnau M, Ledent G, Jangoux M, Dubois P (1996) Seasonal and geographical variations of heavy metal levels, and kinetics of lead accumulation in the asteroid *Asterias rubens,* a key species of North Sea littoral ecosystems. Scientific Symposium on the 1993 North Sea Quality Status Report, p. 105

Annexe II. Participation à des réunions scientifiques.

L'équipe impliquée dans la réalisation de ce contrat a présenté, pendant ces quatre années, les résultats obtenus dans le cadre du présent projet à l'occasion de six conférences internationales.

Colloque international "Indicateurs de la qualité du milieu marin", Carry-Le-Rouet, France. November 20-22 1992.

- Communication orale: "Distribution des polluants métalliques chez l'astérie *Asterias rubens*, espèce-clef des biotopes littoraux de la Mer du Nord"

8th International Echinoderm Conference. Dijon, France. September 06-10 1993.

- Communication orale: "Heavy metals in the asteroid *Asterias rubens* and its prey *Mytilus edulis*: geographical variations in four selected North Sea biotopes".

Scientific Symposium on the 1993 North Sea Quality Status Report. Ebeltoft, Denmark, April 18-21 1994.

- Communication orale: "Lead contamination of *Asterias rubens*, a key-species of North Sea littoral ecosystems".

Fourth European Echinoderms Colloquium. London, United Kingdom, April 10-13 1995.

- Communication orale: "Experimental cadmium contamination of *Asterias rubens*: body distribution of the contaminant and effects on the concentration of essential metals"
- Poster: "Why should the asteroid *Asterias rubens* be included in biomonitoring programmes? A case study on the lead contamination of North Sea littoral biotopes".

9th International Echinoderm Conference. San Francisco, USA. August 5-9 1996.

- Communication orale: "Value of *Asterias rubens* (Asteroidea) in bioindicating heavy metal contamination in naturally-occurring conditions".

84th ICES Statutory Meeting. Reykjavik, Iceland. September 27 - October 4 1996.

- Communication orale: "*Asterias rubens* (Echinodermata, Asteroidea) in polluted Sørfjord (SW Norway): molecular biomarkers and bioavailability of heavy metals".

Annexe III. Travaux académiques

- Deux travaux de fin d'études:

Aboutboul P (1993-1994) Ecotoxicologie du plomb en Mer du Nord: distribution et dynamique d'accumulation du plomb chez *Asterias rubens* (L.)

Nguyen QA (1994-1995) Effets du plomb sur la squelettogenèse d'*Asterias rubens* (Echinodermata, Asteroidea)

- Une thèse de doctorat:

Temara A (1992 - 1996) Distribution, flux et effets biologiques des contaminants métalliques chez *Asterias rubens* (Echinodermata, Asteroidea)

Annexe IV. Texte des articles

Factors influencing the concentrations of heavy metals in the asteroid *Asterias rubens* L. (Echinodermata)

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Submitted Manuscript

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Abstract

Seasonal, geographical, anatomical and sex-related variations of heavy metal concentrations were investigated in the asteroid *Asterias rubens* collected in four representative biotopes of the NE Atlantic at four periods of an annual gametogenic cycle. Zn, Pb, Cd, Fe, Cr, Cu, Ti and Hg concentrations were measured in the gonads, the central digestive tract, the pyloric caeca, the body wall and the skeleton. According to multi-way analyses of variance, the factors considered in the present study accounted for a large proportion (59 - 93%) of total variability of concentrations of all metals but Hg (2%). The body compartment appeared as the most important factor (9.7 up to 88% of total variability). Interactions between the body compartment and the sampling location factors accounted for up to 29% of total variability indicating that bioavailability of heavy metals to asteroids differed from site to site. Weak interactions between the body compartment and the sampling period factors indicated that bioaccumulation was synchronous within the asteroid body. Concentrations of groups Ib and IIb metals and Fe were significantly higher in the pyloric caeca. The other elements were preferentially accumulated in the skeleton. Most bioconcentration factors (BCF) towards preys were lower than 1 in asteroids feeding on filter-feeders (ascidians and mussels), suggesting that most heavy metals were not biomagnified. On the contrary, most BCF in asteroids feeding on grazers (gastropods) were higher than 1. Sex-related differences in the gonads were significant for Zn, Cd, Fe and Cr concentrations (variation ratio: 1.7 to 3.3).

Key words: Heavy metals - Field survey - *Asterias rubens* - Bioconcentration factor

Introduction

Predator asteroids have been demonstrated to affect both qualitatively and quantitatively numerous benthic communities (Menge 1982, Hostens & Hammerlynck 1994). In environments contaminated by heavy metals, such communities are an important target of heavy metals (Karbe et al. 1994) and predator asteroids are likely to affect heavy metal flows in them. In particular, the common NE Atlantic asteroid *Asterias rubens* was shown through experimental exposures to accumulate several metals (Sr, Pu, Cd, Se, Hg, Mn, Pb) that are translocated between body compartments (Binyon 1978, Guary et al. 1982, Bjerregaard 1988, Besten et al. 1989, Besten et al. 1990, Sorensen & Bjerregaard 1991, Rouleau et al. 1993, Hansen & Bjerregaard 1995, Temara et al. in press, Temara et al. in prep). It is noteworthy that, at least for Cd and Pb, heavy metal bioaccumulation in *A. rubens* is directly related to the environmental concentrations of these metals (Bjerregaard 1988, Temara et al. in prep). It has been reported that heavy metal bioaccumulation rates vary according to the body compartment (Bjerregaard 1988, Temara et al. in prep) but other possible sources of variations like season or sex have been uninvestigated.

The aim of the present paper is to determine the relative importance of sources of variation (body compartment, season, sex, type of preys) for the concentrations of eight heavy metals (Cd, Pb, Hg, Zn, Fe, Cr, Cu, Ti) in four asteroid populations living in different biotopes of the Southern Bight of the North Sea and the English Channel.

Materials and methods

Sampling. Individuals *Asterias rubens,* Linnaeus 1758, were collected by SCUBA diving or sea shore fishing at three characteristic stages of the asteroid gametogenic cycle: prespawning (April 1992, 1993), postspawning (August 1992), gametogenesis (January 1993) in an open estuarine site (Wemeldinge, Zealand, The Netherlands), a closed estuarine site (Scharendijke, Zealand, The Netherlands), an intertidal site (Audresselles, Pas-de-Calais, France) and an offshore site (50°45'N - 1°20' E, off Boulogne, Pas-de-Calais, France). All these sites harbour stable asteroid populations. Ten specimens belonging to the largest size-class in the considered site (arm length - measured from the tip of the longest arm to the opposite interradius - ≥ 12 cm) were collected at each sampling. Asteroids were carried back to the laboratory in an aerated aquarium filled with water from the sampling site and were dissected the very same day. Four body compartments were separated: the gonads, the central digestive tract, the pyloric caeca and the body wall (with the ambulacral structures). The skeleton was then separated from a part of the body wall by proteinase cleaning (Dubois & Jangoux 1985). The sex of each asteroid was determined by histology: a piece of gonad from all the collected asteroids (except those in postspawning stage) was taken, weighed (wet weight, ww) and fixed in Bouin's fluid for 24 h, rinsed in 70 %

Table 1. *Asterias rubens*. Characteristics of the analysed samples. Presented data (means ± SD) are: dry weight (g) of the body compartments, water fraction (%) of the wet body compartments and proportion of the skeleton (sk) in the body wall (% of body wall dry weight). CDT: central digestive tract. Presp.: prespawning; postsp.: postspawning; gametogen.: gametogenesis. gc: gonads reduced as genital cords. n.a.: not analysed. IS: intertidal site. CES: closed estuarine site. OES: open estuarine site. OS: offshore site.

Period	Location	DRY WEIGHT (g) (% water)				
		Body wall	Sk (%)	CDT	Pyloric caeca	Gonads
Presp. 92	IS	16.6 ± 5.0 (66.0 ± 1.6)	64.2 ± 2.4	0.30 ± 0.09 (81.4 ± 1.2)	2.2 ± 0.8 (78.0 ± 5.6)	4.7 ± 2.5 (78.8 ± 1.1)
	OS	9.4 ± 2.9 (68.3 ± 2.3)	54.2 ± 6.4	0.28 ± 0.11 (79.3 ± 1.7)	1.2 ± 0.6 (71.8 ± 2.0)	0.2 ± 0.1 (85.2 ± 1.3)
	CES	25.8 ± 4.8 (70.5 ± 1.3)	57.6 ± 4.9	0.52 ± 0.10 (81.5 ± 1.2)	7.8 ± 2.8 (70.7 ± 2.7)	13.5 ± 3.0 (79.0 ± 1.0)
	OES	26.5 ± 3.9 (67.1 ± 1.2)	64.7 ± 2.5	0.62 ± 0.12 (80.9 ± 0.59)	3.9 ± 1.3 (72.5 ± 2.2)	7.6 ± 4.5 (81.1 ± 0.8)
Postsp.	IS	20.6 ± 2.9 (66.6 ± 1.7)	62.0 ± 3.3	0.28 ± 0.04 (80.1 ± 0.8)	8.5 ± 2.8 (65.4 ± 1.4)	gc
	OS	8.9 ± 1.9 (66.9 ± 2.5)	59.2 ± 4.0	0.18 ± 0.06 (79.5 ± 0.7)	2.0 ± 1.4 (68.0 ± 5.3)	gc
	CES	27.7 ± 5.8 (67.9 ± 1.1)	61.0 ± 2.9	0.42 ± 0.08 (80.8 ± 1.4)	8.9 ± 2.6 (65.7 ± 3.6)	gc
	OES	22.2 ± 5.9 (68.1 ± 0.7)	64.0 ± 1.8	0.42 ± 0.08 (79.9 ± 1.4)	4.7 ± 3.9 (65.1 ± 2.7)	gc
Gametogen.	IS	20.7 ± 7.4 (67.5 ± 2.1)	n.a.	0.34 ± 0.12 (81.5 ± 0.7)	2.6 ± 2.2 (70.7 ± 2.2)	3.3 ± 1.6 (79.5 ± 1.6)
	OS	n.a.	n.a.	n.a.	n.a.	n.a.
	CES	11.3 ± 3.0 (70.1 ± 4.5)	n.a.	0.44 ± 0.12 (81.3 ± 0.7)	4.9 ± 1.9 (68.1 ± 2.5)	1.3 ± 1.0 (79.8 ± 1.8)
	OES	14.6 ± 6 (66.1 ± 1.1)	n.a.	0.37 ± 0.15 (81.6 ± 0.9)	2.9 ± 4.6 (72.1 ± 3.2)	3.5 ± 1 (78.7 ± 2.0)
Presp. 93	IS	30.1 ± 3.4 (65.6 ± 1.4)	61.8 ± 2.6	0.45 ± 0.05 (80.5 ± 0.5)	4.0 ± 1.2 (75.3 ± 3.0)	5.4 ± 3.7 (82.4 ± 3.1)
	OS	12.9 ± 3.0 (65.7 ± 1.8)	59.5 ± 9.9	0.36 ± 0.10 (80.7 ± 2.3)	2.6 ± 1.0 (76.5 ± 6.4)	2.8 ± 2.55 (78.9 ± 9.1)
	CES	29.1 ± 5.2 (64.8 ± 12.5)	58.9 ± 5.3	0.55 ± 0.10 (83.0 ± 4.2)	9.2 ± 3.5 (70.1 ± 5.2)	6.0 ± 2.2 (82.1 ± 1.7)
	OES	20.7 ± 7.4 (65.2 ± 1.2)	61.9 ± 3.5	0.48 ± 0.16 (81.3 ± 0.7)	3.0 ± 2.4 (73.4 ± 3.5)	2.8 ± 3.0 (83.7 ± 2.1)

ethanol, dehydrated, embedded in Paraplast and cut into 5 µm-thick sections. Alcian blue (pH 2.6) followed by Groat's hematoxylin and phloxine was used as staining procedures (Ganter & Jollès 1969-1970).

At each sampling, 20 specimens of the specific prey of the studied population were collected simultaneously to the asteroids. The population in the open estuarine site feeds on ascidians (*Ciona intestinalis* L.), the population in the closed estuarine site feeds on gastropods (*Crepidula fornicata* L.) and that in the intertidal site feeds on bivalves (*Mytilus edulis* L.). No preferential prey could have been determined for the offshore population.

All samples of pyloric caeca, gonads, body wall and skeleton were divided in two parts. For each sampling, ten preys (soft tissues) and one part of the divided samples were weighed (ww) and stored in acid-washed, hermetically sealed, polyethylene containers at -40°C until further processing for Hg analysis. Parallelly, ten preys (soft tissues), the other part of the divided asteroid samples and the samples of central digestive tract were dried at 100°C for 48h, weighed (dry weight, dw) and stored until Cd, Pb, Ti, Cr, Cu, Zn and Fe analysis. (The central digestive tract samples were not divided because of their very low mass). Characteristics of the analysed samples are presented in Table 1.

Metal analysis. Hg analysis was carried out according to Bouquegneau (1973) with a MAS50B spectrophotometer. The reproductibility of the methodology was checked using an internal standard (homogenized powder of asteroid body wall). Hg content of 3 internal standard samples was analysed with each sample set. Hg concentration in those samples was 0.085 ± 0.021 µg Hg g^{-1} dw (n=15). A certified material (tuna homogenate, IAEA-350) was analysed in order to check the accuracy of the methodology (Table 2). A dilution factor (50) was applied in order to obtain a solution with a concentration similar to that of the samples. Detection limit (mean + 3 SD of the blanks) equalled 0.0132 µg Hg. Concentrations were expressed as µg Hg g^{-1} dw using the ratio between dw and ww (Table 1).

Sample preparation for Cd, Pb, Ti, Cr, Cu, Zn and Fe analysis was carried out according to Warnau et al. (1995). Metal concentrations were measured by atomic emission spectrometry (ICPS-Jobin Yvon 38+). A certified material (*Mytilus edulis*, CRM n° 278, Griepink & Muntau 1988) was analysed along with the experimental samples to check the accuracy of the methodology (Table 2). Detection limits (mean + 3 SD of the blank) equalled: 2 µg Zn l^{-1}, 1 µg Cd l^{-1}, 15 µg Cu l^{-1}, 14 µg Pb l^{-1}, 4 µg Fe l^{-1}, 1 µg Cr $^{-1}$, 1 µg Ti l^{-1}.

Data analyses. Statistical analyses of the data included 1-, 2- and 3-way ANOVA and the mean multiple comparison test of Tukey. They were performed using the Systat software. The variability explained by each factor was derived from the sum of squares of ANOVA. The level of significance was set at $\alpha = 0.05$ (Zar 1984). Measurements below detection limit were replaced by half of this value for data analysis (Black 1991). Indirect bioconcentration factors were calculated by dividing the mean metal concentrations (MMC) in different body compartments of the asteroids by the MMC in the soft tissues of the preys (*i.e.* the ingested tissues). Direct bioconcentration factors were calculated using the data of Kersten et al. (1988) and NSTF (1993) by dividing the MMC in the different body compartments of the asteroids by the MMC in the sea water. Stocks of metals associated with *A. rubens* and annual flows of metals in asteroid populations of the Eastern Scheldt were calculated using mean asteroid body concentrations (average of MMC in the different body compartments corrected according to relative mass of compartments), metal concentrations in *C. fornicata* from the present study and data on *A. rubens* biomass and consumption from Hostens & Hamerlynck (1994).

Results

Total variability of heavy metal concentrations in *Asterias rubens*

The whole set of concentration data has been deposited in the database of the International Council for the Exploration of the Sea and that of the Management Unit of the North Scheldt and North Sea Mathematical Model (Belgium) (Reference: MUMM 94 CF. BE). The whole set of data was analysed by 3-way ANOVA (Table 3). (The skeleton was not taken into account in the latter analysis since metal concentrations in the body wall are directly dependent on metal concentrations in the skeleton). These analyses showed that among the considered factors of variation (body compartment, sampling period, sampling location), the body compartment was the predominant factor for all elements either as a single factor or in interaction with sampling location. The period of the gametogenic cycle did affect significantly the concentrations of all heavy metals but Hg, though weakly. Significant and important (from 8 up to 29% of the total variation) interactions between the body compartment and the sampling location factors were detected for Zn, Cd, Fe, Cr and Cu concentrations, indicating that bioavailability of these metals to asteroids differed from site to site. Interactions between the body compartment and the sampling period factors were of rather low importance (≤ 10.5%) indicating that bioaccumulation was almost synchronous in the different body compartments. Triple interactions were significant for Zn, Cd, Cr, Cu and Ti concentrations and accounted for a large proportion of Cd and Cr concentration total variations. The factors considered in the present study accounted for most of Pb concentration variability in *A. rubens* (only 6.8% associated to residual variability). They accounted for most of Zn, Cd, Fe, Cr, Cu and Ti concentration variability (59 up to 78%), but accounted for only 26% of total Hg concentration variability.

In the skeleton, variability of heavy metal concentrations was assessed separately using 2-way ANOVA (Table 4). Sampling location was the predominant factor for Pb, Cd, Cr, Cu and Ti concentrations while period of the gametogenic cycle accounted for most of variations of Zn and

Fe concentrations. Uninvestigated factors accounted for important proportion (25 up to 90% of total variability) of Zn, Pb, Cd, Fe and Hg concentrations in the skeleton.

Heavy metal distribution in *A. rubens*

Body distribution of mean metal concentrations are presented in Table 5. Heavy metal concentrations varied significantly between the body compartments (3-way ANOVA, $p_{\text{compartment factor}}$ always ≤ 0.0001). Hg concentrations were significantly higher in the pyloric caeca and the gonads; Zn, Cd, Fe and Cu concentrations were significantly higher in the pyloric caeca; Pb and Ti were significantly more concentrated in the skeleton; Cr was significantly more concentrated in the body wall and the skeleton.

Percentage loads for each of the studied elements in different body compartments are shown in Table 6 (distribution of Hg loads is not presented since this element was not measured in the central digestive tract). The main part of Cu and Fe total loads was in the pyloric caeca. For the other elements, maximal loads were observed in the body wall. However, percentage loads of Cd, Zn and Cr in the pyloric caeca were important. Pb and Ti were almost exclusively stored in the body wall, more precisely in the skeleton. Within the body wall, the proportion of each element stored in the skeleton increased in the following order: Fe (12%), Hg (16%), Cu (28%), Zn (33%), Cr (64%), Cd (74%), Ti (97%) and Pb (99%). Since the skeleton accounted for about 60% of the total body wall (w/w) (Table 1), Fe, Hg, Cu and Zn appeared to be mostly distributed in the non mineralized part of the body wall; distribution of Cr was balanced; Cd, Ti and Pb were mostly located in the mineralized part of the body wall.

Bioconcentration factors of metals in *A. rubens* towards either sea-water (direct BCF) or the asteroid preys (indirect BCF) were calculated. Direct BCF were always very high (Table 7). Values ranged from $9\ 10^2$ for Fe in the skeleton to $4\ 10^5$ for Zn and Hg in the pyloric caeca. Most indirect BCF towards suspension-feeder preys (i.e. *Mytilus edulis* and *Ciona intestinalis*) (Table 8) were lower than 1 indicating that biomagnification hardly takes place in these food chains. However, there were a few exceptions: Pb, Cd and Zn in the body wall and the skeleton; Cu, Cd, Hg and Zn (group I and IIb metals) in the pyloric caeca, Zn in the gonads. On the contrary, most indirect BCF in the population from the closed estuarine site, which feeds on the grazer *Crepidula fornicata*, were higher than 1.

Table 9. *Asterias rubens*. Variability of mean heavy metal concentrations in the body compartments according to the period of the gametogenic cycle: max and min mean concentrations ± SD (µg metal g^{-1} dw, n=10) and ratio between them in brackets. ns: means not significantly different. n.a.: not analysed.

	Hg	Pb	Cr	Ti	Fe	Cu	Cd	Zn
Body wall	0.09 ± 0.05	6.0 ± 1.5	0.74 ± 0.10	0.57 ± 0.09	53 ± 36	2.7 ± 0.41	0.77 ± 0.28	211 ± 130
	(1.8)	(1.5)	(1.2)	(2)	(1.4)	(1.5)	(1.3)	(1.6)
	0.05 ± 0.05	4.0 ± 0.61	0.01 ± 0.25	0.29 ± 0.10	39 ± 16	1.8 ± 0.31	0.57 ± 0.21	132 ± 60
Skeleton	0.03 ± 0.01	9.68 ± 0.45	1.02 ± 0.09	1.01 ± 0.08	10.3 ± 10.2	1.2 ± 0.13	0.91 ± 0.13	105 ± 83
	(ns)	(1.4)	(6.4)	(1.8)	(ns)	(1.6)	(1.2)	(ns)
	0.03 ± 0.01	7.84 ± 0.40	0.16 ± 0.07	0.56 ± 0.06	8.0 ± 5.0	0.73 ± 0.18	0.74 ± 0.14	83 ± 60
Pyloric caeca	0.169 ± 0.139	0.78 ± 0.53	0.70 ± 0.31	0.047 ± 0.085	294 ± 248	66 ± 5.5	2.7 ± 1.3	284 ± 365
	(ns)	(2.5)	(1.9)	(3.9)	(ns)	(2.7)	(1.7)	(1.7)
	0.107 ± 0.155	0.31 ± 0.24	0.37 ± 0.25	0.012 ± 0.0001	368 ± 336	24 ± 32	1.5 ± 1.3	167 ± 178
CDT	n.a.	0.85 ± 0.58	0.74 ± 0.30	0.29 ± 0.66	126 ± 88	10.7 ± 9.8	0.65 ± 0.42	130 ± 43
	(n.a.)	(1.8)	(1.4)	(15)	(1.3)	(1.4)	(7.2)	(1.6)
	n.a.	0.46 ± 0.28	0.53 ± 0.15	0.02 ± 0.02	90 ± 37	7.8 ± 2.6	0.09 ± 0.09	79 ± 20
Gonads	0.13 ± 0.20	0.82 ± 0.51	0.59 ± 0.41	0.02 ± 0.05	46 ± 59	5.5 ± 2.2	0.20 ± 0.22	170 ± 103
	(ns)	(ns)	(1.6)	(ns)	(1.2)	(1.2)	(6.7)	(1.7)
	0.09 ± 0.06	0.69 ± 0.2	0.36 ± 0.22	0.01 ± 0.02	37 ± 21	4.5 ± 1.4	0.03 ± 0.05	102 ± 72

Variations between periods of the gametogenic cycle

Significant variations of metal concentrations with the sampling period might result from variations during the annual cycle of environmental metal bioavailability, variations of body compartment mass or a combination of both factors. During the investigated period, the body compartment masses did vary (Table 1). Ratios between maximal and minimal mass of the body wall and the central digestive tract averaged 1.3 and those of the pyloric caeca 1.6; the gonads varied from tiny genital cords to fully developed organs (8.6 g dw). Therefore, variation of the body compartment mass during the studied annual cycle could account for a part of the heavy metal concentration variability described hereafter and in Table 9.

In the pyloric caeca, the concentrations of all metals but Hg varied significantly (2-way ANOVA) according to the period of the gametogenic cycle. Concentrations were higher during the prespawning periods (1992 and 1993), intermediate during gametogenesis and lower during the postspawning period (Tukey test on mean concentrations for the different sampling periods) except Fe concentrations which could not be discriminated on a seasonnal basis by the Tukey test. Ratios between maximal mean concentrations during the prespawning periods and minimal mean concentrations during the postspawning period were about 1.8 (Zn, Cd, Cr), 2.6 (Pb, Cu) and 3.9 (Ti).

In the central digestive tract, concentrations of all heavy metals varied significantly according to the period of the gametogenic cycle (2-way ANOVA, $p_{\text{sampling period}}$ always ≤ 0.03). Zn concentrations were significanly higher in asteroids collected during the postspawning period; Cu concentrations were significanly higher in asteroids collected during gametogenesis. Concentrations of all other metals were significanly higher in asteroids collected during the prespawning 1992 period. Ratios between maximal and minimal mean concentrations varied between 1.3 and 1.8 for all metals, except for Cd (7.2) and Ti (15).

In the gonads, the concentrations of all metals but Hg varied significantly (2-way ANOVA) according to the period of the gametogenic cycle. Concentrations were significantly higher in one of either prespawning periods (Tukey test) except Pb and Ti concentrations which could not be discriminated on a seasonnal basis by the Tukey test. Ratios between maximal and minimal mean concentrations varied between 1.2 and 1.7 for all metals, except for Cd (6.7).

In the skeleton, the concentrations of all metals but Zn and Fe varied significantly (2-way ANOVA) according to the period of the gametogenic cycle. Concentrations were significantly higher during the prespawning 1993 period. Ratios between maximal and minimal mean concentrations varied between 1.2 and 1.8 for all metals, except for Cr (6.4).

In the body wall, heavy metal concentrations varied significantly according to the period of the gametogenic cycle (2-way ANOVA, $p_{\text{sampling period}} \leq 0.03$). Periods during which maximal concentrations occured differed according to the metal. Ratios between maximal and minimal mean concentrations varied between 1.2 and 2.

Table 10. *Asterias rubens*. Variability of mean heavy metal concentrations in body compartments according to the sampling location: max and min concentrations ± SD (µg metal g^{-1} dw, n=10) and ratio between them in brackets. ns: means not significantly different. n.a.: not analysed.

	Hg	Pb	Cr	Ti	Fe	Cu	Cd	Zn
	0.09 ± 0.06	6.2 ± 1.8	0.81 ± 0.09	0.48 ± 0.11	61 ± 23	2.7 ± 0.91	0.87 ± 0.31	312 ± 134
Body wall	(1.8)	(1.3)	(1.2)	(1.5)	(2.1)	(1.4)	(1.6)	(2.8)
	0.05 ± 0.04	4.7 ± 1.1	0.66 ± 0.10	0.32 ± 0.13	29 ± 7.4	1.9 ± 0.51	0.95 ± 0.15	113 ± 38
	0.03 ± 0.01	9.1 ± 1.1	0.76 ± 0.39	0.76 ± 0.17	14.1 ± 7.3	1.1 ± 0.12	0.91 ± 0.3	177 ± 98
Skeleton	(ns)	(1.1)	(ns)	(1.1)	(2.8)	(1.1)	(1.1)	(3.2)
	0.03 ± 0.01	8.4 ± 0.9	0.71 ± 0.33	0.69 ± 0.18	5.1 ± 2.1	0.96 ± 0.24	0.79 ± 0.09	54 ± 12
	0.23 ± 0.17	0.79 ± 0.46	0.99 ± 0.32	0.06 ± 0.09	675 ± 306	90.6 ± 42.7	3.07 ± 1.7	553 ± 386
Pyloric caeca	(2.6)	(2.7)	(4)	(6)	(5.5)	(8.2)	(2.6)	(8)
	0.09 ± 0.08	0.29 ± 0.28	0.25 ± 0.18	0.01 ± 0.00	117 ± 44	11.0 ± 3.6	1.2 ± 0.82	69 ± 26
	n.a.	0.84 ± 0.65	0.79 ± 0.24	0.21 ± 0.48	114 ± 84	11.9 ± 5.2	0.68 ± 0.48	123 ± 27
CDT	(n.a.)	(1.8)	(1.6)	(10)	(1.2)	(1.9)	(3.1)	(1.2)
	n.a.	0.46 ± 0.25	0.50 ± 0.08	0.02 ± 0.01	92 ± 28	6.3 ± 2.3	0.22 ± 0.22	101 ± 23
	0.16 ± 0.09	0.94 ± 0.48	0.73 ± 0.79	0.04 ± 0.09	89 ± 107	7.7 ± 4.1	0.28 ± 0.24	202 ± 93
Gonads	(2.3)	(2.6)	(1.8)	(4)	(2.5)	(1.7)	(4.7)	(1.6)
	0.07 ± 0.05	0.59 ± 0.32	0.41 ± 0.16	0.01 ± 0.00	34 ± 15	4.5 ± 0.8	0.06 ± 0.05	129 ± 93

Comparisons of metal loads indicated that at gonad maturity, Cu and Fe were principally (*ca.* 75 % of total metal load of the asteroid) accumulated in the gonads while the main proportion of total metal loads of the other elements were in the body wall. After gamete emission, Cu, Fe and Cd were principally accumulated in the pyloric caeca (55 to 70% of total metal load of the asteroid). Those variations were observed regardless of sampling site.

Variations between populations

In the skeleton, the central digestive tract, the pyloric caeca and the body wall, heavy metal concentrations varied significantly according to sampling location (2-way ANOVA, $p_{\text{sampling location}} \leq 0.04$, 0.03, 0.001 and 0.0002, respectively), with the exceptions of Hg concentrations in the skeleton ($p_{\text{sampling location}}=0.3$) and Fe concentrations in the central digestive tract ($p_{\text{sampling location}}=0.4$). Ranking of concentrations according to sampling location differed according to the metal. Ratios between maximal and minimal mean concentrations in the skeleton, the central digestive tract and the body wall varied between 1.1 and 3.2, except for Ti concentrations in the central digestive tract (10). In the pyloric caeca, ratios between maximal and minimal mean concentrations were higher (2.6 to 8.2) (Table 10).

In the gonads, concentrations of all heavy metals but Hg and Zn varied significantly according to the sampling location (2-way ANOVA). Pb concentrations were significantly higher in asteroids collected in the intertidal site while concentrations of all other metals were significantly higher in asteroids collected in the offshore site. Ratios between maximal and minimal mean concentrations varied between 1.6 and 4.

The four studied populations live in biotopes with contrasted hydrogeographic parameters. Moreover, those populations feed on different preys. Taking into acount the quantity of food daily ingested by an adult asteroid and the concentrations measured in the preys, quantities of metals daily ingested by an asteroid were calculated (Table 11). These quantities were significantly different from one population to another, except for Cu (p=0.08).

Mean concentrations in each body compartment were ranked according to the different sampling locations and ranks were compared to quantities of metal daily ingested. In the central digestive tract, ranks of Pb, Ti and Cr concentrations in asteroids fitted the rank of sampling sites according to quantity of dietary metal, while those of Zn, Hg, Fe, Cd and Cu concentrations did not. In the pyloric caeca, the gonads, the skeleton and the body wall, no concentration rank fitted the rank of sampling site according to quantity of dietary metal, with the exception of that of Pb.

Variations between male and female asteroids

The differences between heavy metal concentrations in male and female gonads were tested using 1-way ANOVA. Four out of the eight studied metals showed significant differences according to the sex of the individuals, Zn concentrations showing the most pronounced variation (Table 12).

Discussion

Heavy metals were found to be selectively distributed among the body compartments of *A. rubens.* Heavy metals of group I and IIb of the Periodic table and Fe showed higher concentrations in the non-calcified compartments while other elements were concentrated in the body wall and were principally located in the skeleton. In particular, Pb and Ti were highly concentrated in the skeleton, showing concentrations which were 1 order of magnitude higher than those in the non-calcified compartments. While Pb is indeed well-known as a skeletal seeking element (*see* Temara et al. 1995), little information about Ti is available.

The high incidence of the body compartment type on heavy metal concentrations is of primary importance since *A. rubens* has increasingly been used as a reference species though analysed as a whole organism (e.g. Everaarts & Fisher 1989, Vynke et al. 1991). For example, Vynke et al. (1991) reported Hg concentrations from 160 up to 240 ng Hg g^{-1} dw in the whole body of *A. rubens* specimens sampled from 1985 to 1989 in the Southern Bight of the North Sea. Such variability was lower than the variability between compartments reported in the present study (30 to 150 ng Hg g^{-1} dw). However, it is worth noting that our samples, taken 8 years later in the same general area, showed lower Hg concentrations regardless of the body compartment, confirming downward trends reported in the North Sea (NSTF 1993). Similarly, the variability of Cd concentrations according to the body compartment measured in the present study was higher than geographical variations reported by Everaarts et al. (1990) analyzing whole *A. rubens.*

Zn was more concentrated in the pyloric caeca than in any other compartment. The pyloric caeca are storage organs that redistribute nutrients over the rest of the body and are therefore a site of high metabolic activity (Oudejans et al. 1979). Zn is implied in many biological reactions as a cofactor for a number of enzymes, which could explain such high concentrations in this compartment. As shown in the present study, female gonads were characterized by Zn concentrations that highly exceeded the concentrations in male gonads. This sex-related difference was also observed by Besten et al. (1991). These authors showed that high molecular mass proteins in the *A. rubens* pyloric caeca contain Zn. They hypothesized that Zn in the gonads originates from the pyloric caeca where it had been stored bound to metallothionein-like proteins. According to Voogt et al. (1985), precursors of glycoproteinic yolk substances which are deposited in oocytes during vitellogenesis originate from the pyloric caeca. As these precursors are high molecular mass proteins, it can be hypothesized that they are rich in Zn and that they translocate it with them to the gonads, explaining the sex-related difference in Zn concentrations in this compartment.

Hg concentrations generally did not show any significant variation during the studied annual cycle regardless of the considered body compartment. This implies a Hg translocation from the pyloric caeca to the gonads since the mass of those compartments vary significantly during the gametogenic cycle (Jangoux & Vloebergh 1973, present study). The pyloric caeca presented the highest Hg concentrations in all studied sites. This is in accordance with results of Sorensen & Bjerregaard (1991) showing that the pyloric caeca present the highest Hg accumulation rate in asteroids. Studies on the uptake of Hg by *A. rubens* revealed that this metal is translocated within the asteroid body under steady state conditions through the haemal system (Rouleau et al. 1993) as this system does for nutrients (Broertjes et al. 1980). According to our results, a translocation mechanism is responsible for the accumulation of about 23% of the total Hg body load into the gonads during each prespawning period. This load could then be eliminated with the gametes. The residual body load is stored in the pyloric caeca where it could be trapped either by metallothioneins or by Se interaction (Besten et al. 1990, Sorensen & Bjerregaard 1991). Elimination of other metals through spawning could occur in *A. rubens*, though in a lower proportion than for Hg.

These data on the distribution of heavy metals in the asteroid body are in accordance with and expand the few available reports concerning *A. rubens*. [To our knowledge, only few authors - Bjerregaard (1988), Besten et al. (1991), Sorensen & Bjerregaard 1991 - separated the body compartments for metal analysis]. However, these reports did not precise neither the size of the collected individuals nor the period of collection. This could explain the lower Cd concentrations reported for the body wall by these authors (there is a well-defined positive relationship between Cd concentration in the body wall and size in *A. rubens*, Temara et al. 1996).

The indirect BCF along the food chain "suspended matter-mussel-asteroid" are presented in Table 13. All indirect BCF of mussels are lower than 1 indicating that no biomagnification occurs. On the contrary some indirect BCF of asteroids are higher than 1 (Cu, Zn, Cd) while no biomagnification occurs for Ti and Cr in this food chain. In particular, Ti which is a physiologically inert element, is highly adsorbed on the suspended matter whereas it is not bioaccumulated. Although some examples of radionuclide biomagnifications involving asteroids have been reported (Guary et al. 1982), biomagnification of heavy metals is a rare process in the marine environment (Bouquegneau & Joiris 1988) and appears to be limited in *A. rubens*.

A. rubens plays a crucial ecological role within the mobile epifauna of the eastern Scheldt (the area including the 2 "Zealand sites" of the present study) and accounts for over 50% of the total epibenthic production and consumption in this habitat (Hostens & Hamerlynck 1994). Similarly, according to the present study, *A. rubens* plays a significant role in the global heavy metal cycle in this environment (Table 14).

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Allometric Variations in Heavy Metal Bioconcentration in the Asteroid *Asterias rubens* (Echinodermata)

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Résumé: Cent astéries *Asterias rubens*, représentatives de la gamme de taille (1 - 16 cm) de la population étudiée ont été prélevées dans l'Escault occidental en 1992. Après mesure de leur taille, les astéries ont été disséquées et les concentrations en Zn, Pb, Cd, Fe, Cr, Cu et Ti ont été mesurées dans le système digestif et le tégument. Les relations entre taille et concentrations ont été modèlisées par régressions linéaires après transformations adéquates des données. La distribution des fréquences de tailles mesurées dans la population a été décomposée en 4 cohortes, ce qui indique que ces astéries vivent au moins 4 ans. Dans le compartiment "Tégument", les concentrations en Cd et en Cr augmentent avec la taille selon une fonction de puissance et une fonction hyperbolique, respectivement; les concentrations variant de 0,3 à 0,8 µg Cd g^{-1} PS et 0,5 à 3,3 µg Cr g^{-1} PS. Les concentrations des autres éléments étudiés ne varient pas significativement avec la taille des astéries. Dans le compartiment "système digestif", les concentrations en Cr (0,5 - 0,8 µg Cr g^{-1} PS) et en Zn (28 - 195 µg Zn g^{-1} PS) augmentent linéairement avec la taille alors que les concentrations en Cd (0,1 - 2,8 µg Cd g^{-1} PS) augmentent selon une fonction de puissance. Au contraire, les concentrations en Pb (3,8 - 0,3 µg Pb g^{-1} PS) et en Ti (2,6 - 0,02 µg Ti g^{-1} PS) diminuent selon une fonction de puissance inverse. Les concentrations en Fe et en Cu ne varient pas significativement avec la taille. La diminution des concentrations en Pb correspond à une taille (4 - 5 cm) d'initiation de la maturité sexuelle et de croissance somatique rapide. A ce stade, les flux de Pb depuis le système digestif vers les gonades et le squelette seraient accélérés, ce qui diminuerait les concentrations instantanées dans le système digestif. Les variations allométriques étant les plus faibles dans la plus grande classe de taille, c'est cette dernière qui devrait être échantillonnée dans les programmes de biomonitoring utilisant *A. rubens* comme espèce de référence.

Mots clefs: *Asterias rubens* - métaux lourds - allométrie

INTRODUCTION

Bioindicators are commonly used to assess ecosystem contamination by pollutants (Peakall 1992). It is now well established that several of these bioindicators are necessary to give a satisfactory account of the pollution status of the ecosystems under study (see, e.g., Gray 1989). To qualify a species as a bioindicator, several aspects of its biology must be known (see Phillips 1976). One of these aspects is the relationship between pollutant concentrations in the biota and age (Newman and Heagler 1991). The common European asteroid *Asterias rubens* has numerous characteristics of a bioindicator species for heavy metal contamination, *viz.*, effective metal accumulation and sensitivity, wide geographical and bathymetrical distribution, presence in numerous ecosystems, sedentary behavior, large size of adult individuals, and status as key species in several communities (den Besten 1989, 1990; Everaarts et al. 1990; Hayward and Ryland 1990). Yet, the relationships between age and metal concentrations in this organism still need to be investigated in order to assess the bioindicator value of the species. The present study examines the age-metal concentration relationships for seven heavy metals (Zn, Cu, Fe, Cd, Pb, Cr, and Ti) in *Asterias rubens*.

MATERIALS AND METHODS

One hundred specimens of the asteroid *Asterias rubens* (Linnaeus, 1758) representing the whole size range of the population were collected by SCUBA diving in August 1992 in Scharendijke (Zeeland, The Netherlands). At that period, the gonads are extremely reduced (Jangoux and Vloebergh 1973) and the possible variation of metal concentrations due to sex factor is therefore minimal. All asteroids were maintained in sea water from the sampling location until dissection.

The asteroids were first measured (arm length -R- measured from the tip of the longest arm to the opposite interradius). They were then dissected, within the day of sampling, using stainless steel instruments. Two body compartments (the digestive system and the body wall) were separated, finely chopped and dried for 24 hr at 100°C. The dried samples were stored in hermetically sealed polyethylene containers at room temperature until further processing. For analysis, an aliquot of each sample (0.5 g dry weight) was processed according to the method described by Warnau et al. (1995). The concentrations of Zn, Cu, Fe, Cd, Pb, Cr and Ti were measured by atomic emission spectrometry (ICPS-Jobin Yvon 38+). A certified material (*Mytilus edulis*, CRM n° 278, Community Bureau of Reference) was analysed along with the experimental samples in order to check the accuracy of the methodology (Table 1). Detection limits (mean + 3 sD of the blank) equaled: 2 µg Zn / L, 14 µg Pb / L, 1 µg Cd / L, 4 µg Fe / L, 1 µg Cr / L, 2 µg Cu / L, 1 µg Ti / L. Measurements below the detection limit were replaced by half of this value.

Table 1. Metal concentrations ($\mu g\ g^{-1}$ DW) in standard tissues used in the analyses (values in brackets are uncertified).

Metal	Certified (mean ± SD)	Measured (min and max). n = 5
Zn	76 ± 2	77 - 82
Pb	1.91 ± 0.04	1.91 - 1.95
Cd	0.34 ± 0.02	0.31 - 0.37
Fe	133 ± 4	126 - 161
Cr	0.8 ± 0.08	0.87 - 1.04
Cu	9.60 ± 0.16	9.60 - 10.71
Ti	(2 ± 0.2)	2.8 - 3.8

The rejection of outliers was performed according to the test of discordancy described by Black (1991). The goodness of fit of the frequency distribution of the asteroid sizes to a succession of normal distributions was tested by a quasi-Newton algorithm using the MIX 3.0 software (Ichthus Data Systems). The relationships between size and metal concentrations in the considered compartments of the asteroids were fitted using linear regression analysis (least square method). Semi-logarithmic, bi-logarithmic, and inverse transformations of the data were systematically performed to test biologically relevant non-linear relationships (exponential, power, and hyperbolic functions, respectively) (Zar 1984). Examinations of the residuals were systematically performed to check homoscedasticity of the transformed and nontransformed data (Zar 1984). The regression models presented are those showing the best fit (highest determination coefficient) while respecting homoscedasticity of the data. Heavy metal concentrations in the two body compartments of *Asterias rubens* were then compared using a student t-test.

RESULTS AND DISCUSSION

The different-sized asteroids were sampled from a single population at Scharendijke (The Netherlands) in August 1992. The size frequency distribution of the studied population fits a succession of four normal distributions (Fig. 1). The parameters of these distributions were used to define the four size classes considered below (S1: arm length -R- ≤ 5.3 cm; S2: 5.3 cm < R ≤ 7.8 cm; S3: 7.8 cm < R ≤ 10.3 cm; S4: 10.3 cm < R).

Size is usually considered as the most suitable approximation of age in asteroids (Guillou 1983). This hypothesis is supported by population dynamics studies which show that (1) asteroids are continuously growing during their life, and (2) negative growth events are limited. The demographic approach of the present study revealed that the size frequency distribution of the *A.*

rubens population from Scharendijke may be split up into four normal distributions or cohorts. Considering the reproductive strategy of *A. rubens*, Guillou (1983) ascribed to such cohorts an annual value. This would mean that the Scharendijke population showed a mean life-span of 4 yr with a mean size of 12.3 cm for the 4-yr-old individuals. These values are intermediate between extremes described in the literature (see Guillou and Guillaumin 1985). Assuming that an annual value may effectively be assigned to each of the four components of the size-frequency distribution, a linear relationship does exist between the measured size and the putative age of the asteroid. The regressions between size and heavy metal concentrations calculated in the present study may thus be considered as representative of the relationships between the asteroid age and the heavy metal concentrations in the two investigated body compartments.

Heavy metal concentrations were measured in the body wall and the digestive system of these asteroids and plotted against the organism's arm length. Simple linear regression analysis of these data in transformed and nontransformed formats were performed. These analyses indicate that relationships between metal concentration and size vary according to both the compartment and metal (Table 2).

In the body wall, Cd and Cr show a significant increase in concentration with size following a power and hyperbolic function, respectively. Zn and Cu concentrations show significant relationships with size only in the simple linear model (nontransformed data). However, the requirement of homoscedasticity is not fulfilled for these data. No significant relationship was found between Pb, Fe, and Ti concentrations and size.

In the digestive system, Zn and Cr concentrations show significant positive linear relationships with size. Pb and Ti concentrations show decreasing power relationships, and Cd concentrations an increasing power relationship with size. No significant relationship was found between Cu and Fe concentrations and size.

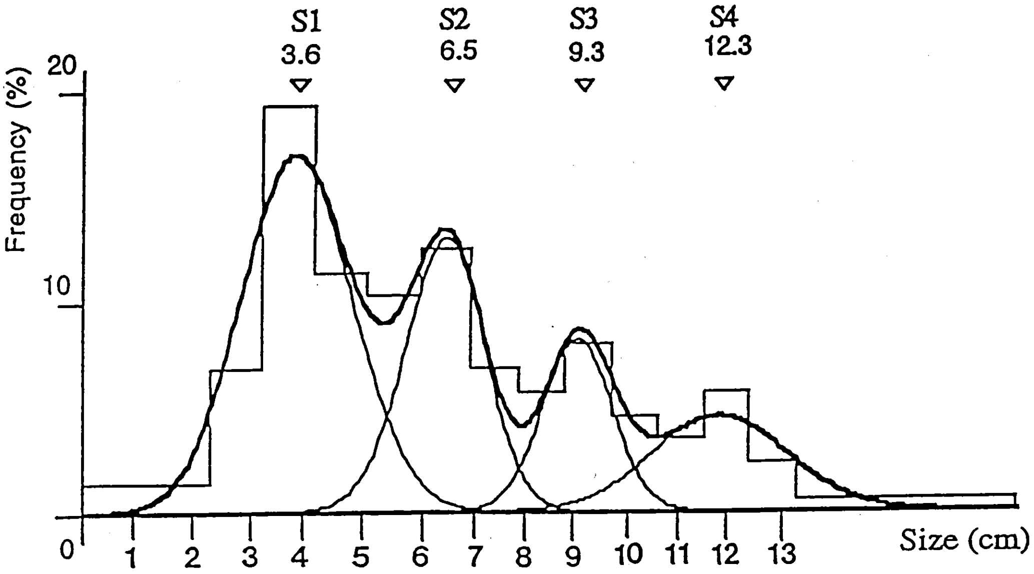

Figure 1. Size-frequency distribution of the *Asterias rubens* population of Scharendijke (August 1992): size-frequency histogram and fitted normal distributions. The bold line is the sum of the fitted normal distributions. Triangles indicate the mean sizes (cm) of the size classes (n = 100).

Table 2. Regression analysis of the relationships between metal concentrations (MC) in the body compartments of *Asterias rubens* and size (arm length -R- in cm). n: number of data used for regression analysis; R^2: determination coefficient; p: probability of the slope; b and a: parameters of the regression models; L: linear model (MC = a+b.R); P: power model ($MC = a .R^b$); H: hyperbolic model ($MC = \{a+b.R\}^{-1}$); n.s.: no significant relationship found ($\alpha = 0.05$); *: simple linear model significant (other models nonsignificant) but heteroscedasticity of the data.

	n	MC range ($\mu g\ g^{-1}$ DW)	Regression model	Regression parameters		Model parameters	
				R^2	p	b	a
Body wall							
Zn	90	40-154	L *				
Pb	90	3.9-8.2	n.s.				
Cd	90	0.3-0.8	P	0.17	$\leq$ 0.0001	0.2	0.4
Fe	90	24-156	n.s.				
Cr	90	0.5-3.3	H	0.23	$\leq$ 0.0001	-0.06	1.6
Cu	90	1.5-3.7	L *				
Ti	90	0.2-0.8	n.s.				
Digestive system							
Zn	90	28-195	L	0.05	0.03	1.9	86.8
Pb	86	0.3-3.8	P	0.57	$\leq$ 0.0001	-0.9	4.0
Cd	88	0.1-2.8	P	0.52	$\leq$ 0.0001	1.3	0.05
Fe	90	190-1800	n.s.				
Cr	85	0.5-0.8	L	0.46	$\leq$ 0.0001	0.03	0.06
Cu	90	9-150	n.s.				
Ti	90	0.02-2.6	P	0.40	$\leq$ 0.0001	-1.2	0.4

Mean metal concentrations (and the associated standard deviations) were calculated either for each size-class (metals whose concentrations are significantly related to size) or for the whole size range (metals whose concentrations do not present a significant relationship with size) (Table 3). Metal concentrations were compared class by class between the two body compartments of the asteroid. All pair comparisons were found to be significantly different (*t* - test; $\alpha = 0.05$) except for Cd in the first size-class.

Table 3. Mean concentrations (M) ($\mu g.g^{-1}$ dry weight) and standard deviations (SD) of heavy metals in the body wall and the digestive system of *Asterias rubens* individuals of the size-classes defined in Fig. 1.

	Body wall			Digestive system		
	Size class	M ± SD	n	Size class	M ± SD	n
Zn	all	69.1 ± 18.4	90	S1	91.0 ± 26.7	42
				S2	104.6 ± 26.8	22
				S3	115.0 ± 36.9	13
				S4	102.4 ± 20.7	13
Pb	all	5.07 ± 0.8	90	S1	1.78 ± 1.29	38
				S2	0.62 ± 0.23	22
				S3	0.63 ± 0.38	13
				S4	0.51 ± 0.16	13
Cd	S1	0.47 ± 0.12	42	S1	0.44 ± 0.55	40
	S2	0.50 ± 0.11	22	S2	0.81 ± 0.56	22
	S3	0.52 ± 0.11	13	S3	0.94 ± 0.59	13
	S4	0.54 ± 0.10	13	S4	1.36 ± 0.73	13
Fe	all	66.2 ± 27.6	90	all	497 ± 279	90
Cr	S1	0.79 ± 0.33	42	S1	0.24 ± 0.32	37
	S2	0.83 ± 0.30	22	S2	0.32 ± 0.13	22
	S3	1.13 ± 0.68	13	S3	0.45 ± 0.16	13
	S4	1.25 ± 0.69	13	S4	0.44 ± 0.15	13
Cu	all	2.16 ± 0.41	90	all	55.9 ± 28.6	90
Ti	all	0.35 ± 0.1	90	S1	0.19 ± 0.41	42
				S2	0.14 ± 0.37	22
				S3	0.03 ± 0.02	13
				S4	0.03 ± 0.02	13

The increasing relationships between Cr and Cd concentrations and size in both asteroid compartments indicate that these metals are continuously accumulated through life and are not, or only poorly, regulated. This is in sharp contrast with the results of Hornung et al. (1991) who showed highly significant negative relationships between Cr concentrations in the tissues of individuals of *Astropecten bispinosus* and the weight of these individuals.

Pb (and to a less extent Ti) concentrations are inversely related to size in the digestive system of *A. rubens*. The highest Pb concentrations were measured in asteroids up to 4-5 cm. This size corresponds to the life stage when sexual maturity and high growth rate are initiated (Mead 1899;

Nauen and Böhm 1979). Therefore, from this life stage, a different allocation of absorbed Pb might occur, *viz.* in the gonads and/or in the body wall. A better understanding of Pb fluxes through the asteroid body during gametogenesis would clarify the role of gonads in the process leading to the concentration lowering in the digestive system. Accumulation of Pb in the body wall is important in large *A. rubens*, being two orders of magnitude larger in S4 size-class than in S1 size-class asteroids (Table 4). We suggest that, once a high growth rate is initiated (i.e., in asteroids reaching 5 cm), Pb fluxes in the asteroid are speeded up, most of the absorbed Pb being finally driven into the body wall, and trapped in the skeleton (95 % of lead in the body wall is actually in the skeleton; Temara et al. unpubl.). This phenomenon would make the instantaneous concentration of metal in the digestive system lower.

Table 4. Pb loads in different compartments of *Asterias rubens* in prespawning and postspawning stages (Temara et al., unpubl.) (µg metal per organ, means ± standard deviations, n= 40). (-): gonads extremely reduced at that period.

Size class	Period	Body wall	Digestive system	Gonads
S4	prespawning	155 ± 41	2.0 ± 1.1	2.3 ± 0.2
S1	postspawning	2 ± 0.5	0.17 ± 0.01	-
S4	postspawning	161 ± 36	2.1 ± 1.4	-

No significant change in Fe and Cu concentrations with size was detected; this observation probably reflects the physiological regulation of these metals (Roesijadi 1992). On the contrary, Zn concentrations show a positive linear relationship with size. It is noteworthy, however, that this relationship is barely significant ($p = 0.03$) and that the size effect is of very low importance ($R^2 = 0.05$). These observations are thus in good agreement with those obtained by Hornung et al. (1991) who found no significant relationship between the concentrations of Cu, Zn, and Fe and weight in *A. bispinosus*.

All significant relationships between size and metal concentrations (except for Cr in the digestive system) show their maximal variation range in the small size class (S1). This means that allometric effects are minimized in the large size-class (S4). We thus propose that this size-class should be sampled if *A. rubens* is used as a bioindicator of metal pollution. If Cr has to be considered, this size range should be further narrowed.

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Experimental cadmium contamination of *Asterias rubens*, L. (Echinodermata)

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Abstract: Asteroids *Asterias rubens* (Linneaus, 1758) were exposed in the field to various concentrations of waterborne or dietary Cd (from contaminated mussels). Cadmium uptake, and subsequent loss, kinetics were studied for 45 d for each. Dietary (110 µg Cd g^{-1} dry wt of the prey) and waterborne (5 and 20 µg Cd l^{-1}) Cd was significantly accumulated in the measured body compartments: the pyloric caeca, the body wall and the skeleton. Waterborne Cd (1 µg Cd l^{-1}) was also significantly accumulated in the body wall but not in the pyloric caeca or the skeleton. Dietary Cd accumulation occurred in all body compartments. In the pyloric caeca, a steady state equilibrium was reached within 20 d, the concentration at steady state ranging from 3 to 9 µg Cd g^{-1} dry wt according to the exposure mode. The body wall and the skeleton accumulated Cd linearly during the exposure period at a rate of 0.07 to 0.25 µg Cd g^{-1} dry wt d^{-1} according to the exposure mode. Cadmium loss kinetics were fitted by inverse exponential functions to all body compartments, except to the skeleton where the loss was generally nonsignificant. Results indicate that there is a Cd flux through the asteroid body from the digestive system to the body wall where Cd is slowly incorporated into the skeleton. Concentration factors calculated for the pyloric caeca fit the range of values previously reported from laboratory experiments and thus validate them in field conditions. Thus *A. rubens* may be considered a valuable bioindicator of Cd contamination. While Cu concentrations did not vary significantly, Zn concentrations increased sharply (42 to 134%) in the pyloric caeca during the first 30 days of exposure regardless of exposure mode. It is suggested that exposure to Cd triggers an entry of Zn which could soften the deleterious effects of Cd and trigger the synthesis of metallothioneins.

Key words: *Asterias rubens* · Field contamination · Cadmium · Uptake kinetics · Loss kinetics

Introduction

Although international commissions have regulated dumping operations in the areas of jurisdiction of contracting countries, heavy metals are still recognized as threatening to continental shelf ecosystems (Newman & McIntosh 1991, NSTF 1993). This is partly due to their conservative nature that keeps them in food webs and sediments. Cadmium is considered as one of the most toxic metals to aquatic biota (Sorensen 1991), and its uptake by marine filter-feeders is well documented (Borchardt 1983, Bebianno & Langston 1991). Surprisingly, little attention has been paid to a main consumer of filter-feeders, the top predator asteroids. Yet, these often form abundant populations that are present in numerous ecosystems (Hayward & Ryland 1990), indicating that the fluxes of metals through these animals could be significant. Furthermore, several asteroids are key species in their communities (Menge 1982). Metal impacts on them will thus affect the whole community.

Cadmium uptake kinetics have been studied in *Asterias rubens* (the main NE Atlantic predator asteroid) exposed in aquaria or mesocosms to high concentrations (from 25 to 400 μg Cd l^{-1}) (Bjerregaard 1988, Besten et al. 1989, 1990). However, modelling of the kinetics was not achieved and therefore steady-state concentrations could not be determined. Cadmium bioaccumulation by the same species exposed to low concentrations (0.0125 to 12.5 μg Cd l^{-1}) was measured in the laboratory for 14 d, but no kinetics were established (Bjerregaard 1988). Also, elimination kinetics of Cd by asteroid body compartments have not been described, though these data are crucial in assessing the fate of the bioaccumulated metal. The aims of the present study were to model the routes of Cd uptake and elimination in the top predator *A. rubens* exposed in the field, and to assess the fate of the contaminant when natural conditions are restored, and to ascertain the effect of Cd exposure on essential metal concentrations.

Materials and Methods

Individuals of *Asterias rubens* (Linneaus, 1758) (size: 6 to 9 cm from the tip of the longest arm to the opposite interradius) were collected in the Eastern Scheldt (Scharendijke, The Netherlands) by SCUBA divers. They were experimentally contaminated in the field from May to August 1994. Two exposure modes of *A. rubens* to Cd were investigated: (1) exposure via sea water and (2) exposure via food.

Contamination from sea water. Contaminations were performed in the field in Plexiglas containers (capacity: 30 l) anchored in the sediment at a depth of 9 m (Warnau et al. 1995). Cadmium was injected into the containers as $Cd(NO_3)_2$ (Titrisol, certified quality, Merck). Asteroids were fed mussels (*Mytilus edulis* L.) *ad libitum*. Water, Cd content, and mussels were renewed twice a day. Three sets of asteroids (n = 27 each) were exposed for 45 d to 3 different

Cd concentrations in sea water (nominal concentrations: 1, 5, and 20 µg Cd l^{-1}). Actual Cd concentrations in the containers were assessed by collecting a small volume (5 ml) of water from 3 control containers (with 15 asteroids each) 2, 8, and 12 h after Cd injection. Water was also sampled in the field to assess background Cd concentration. The water samples were filtered (0.45 µm, glass microfiber filters, Whatman) and acidified (acidifying solution: 65% nitric acid, Suprapur quality, Merck; pH of final solution < 2) immediately after they had been brought to the surface. Cadmium concentrations in these water samples were measured by electrothermal atomic absorption spectrometry with a Varian SpectrAA-300 Zeeman spectrometer equipped with a GTA-96 graphite furnace.

Contamination through food. The chosen prey was the mussel *Mytilus edulis*. A total of 2000 mussels were collected in the Eastern Scheldt (Wemeldinge, The Netherlands) and exposed for 2 m in a closed-circuit aquarium (250 l) to Cd (as $Cd(NO_3)_2$) dissolved in sea water at a nominal concentration of 20 µg Cd l^{-1}. Cadmium concentrations in sea water were daily measured by atomic absorption spectrometry (GBC 906 AA spectrometer) and adjusted when required. Contamination of the asteroids was then performed in the field at 9 m depth, in a polyethylene meshed cage. Over 45 d, 27 asteroids were fed the previously contaminated mussels *ad libitum*. These were replaced by freshly contaminated mussels every 15 d. Mussels were regularly collected in the cages and Cd concentration in their soft tissues was measured.

Decontamination. After Cd exposure by seawater or diet, tested asteroids were placed in polyethylene meshed cages and fed noncontaminated mussels for 45 d.

Controls. Asteroids from the same population were sampled regularly 100 m away from the experimental site to take into account individual fluctuations of Cd concentrations under noncontaminating conditions ('field controls'). In order to assess possible effects of the experimental device, 27 asteroids were placed for 45 d under a Plexiglass container ('container controls') or 90 d in a polyethylene meshed cage ('cage controls') and submitted to the same treatment and sampling schedule as the experimental asteroids except for Cd exposure.

Sample preparation and metal analysis. Samples of 3 asteroids each were collected at regular time intervals in every experimental and control group. Each sampled asteroid was dissected just after collection. The pyloric caeca and the body wall were kept for analysis. As the major part of the experiment was carried out during the postspawning period, gonads were not considered. Processing of the samples and isolation of the skeleton were performed as described by Warnau et al. (1995). The concentrations of metals in the body compartments of the asteroids were measured by atomic absorption spectrometry (GBC 906 AA spectrometer) after acid digestion of the samples. The reproducibility of the methodology was checked using an internal standard (homogenized powder of asteroid body wall). Metal concentrations of 3 samples of the internal standard were analysed with each sample set. The variations of these measurements (n = 15) were low (always within ± 2 SD of the first set mean). A certified material (*Mytilus edulis*, CRM n° 278, Griepink & Muntau 1988) was analysed along with the experimental samples to check the accuracy of the methodology [certified value: 76 ± 2 μg Cd g^{-1} dry wt (mean ± SD), measured values: 75 to 85 μg g^{-1} dry wt, n = 5]. Detection limits (mean + 3 SD of the blank) were 12 μg Cd l^{-1}, 2 μg Zn l^{-1} and 2 μg Cu l^{-1}. Measurements below the detection limit were replaced by half of this value for data analysis (Black 1991).

Data analysis. Significance of the differences between metal concentrations was tested by one-way analysis of variance (ANOVA) and by the Tukey's multiple comparison test (Zar 1984). The variations of Cd concentration in the soft tissues of the contaminated mussels were analysed by simple linear regression to describe Cd uptake and to test the significance of Cd loss when noncontaminating conditions had been restored.

Uptake kinetics of the asteroid body compartments were described using either a simple linear or a nonlinear model. Regressions using both models were systematically performed; statistics of the best fit (highest R^2) are presented. When the linear model showed the best fit, linearity was tested by the linearity test for regression with replication (Zar 1984). Significance of the slopes of the linear regression equations was tested by 1-way analysis of variance (ANOVA). Comparisons between slopes of the linear regression equations were tested either by the bilateral *t*-test (comparison between 2 regression slopes) or by analysis of covariance (ANCOVA) followed by Tukey's multiple comparison test (multiple comparisons between more than 2 regression slopes) (Zar 1984). The nonlinear model was: $C_t - C_0 = (C_s - C_0)(1 - e^{-kt})$; where C_0, C_t, and C_s are the Cd concentrations at times 0, t, and steady-state; k is the uptake rate constant (d^{-1}) (adapted from Whicker & Schultz 1982). Parameters of the equations (C_0, C_s, k) were calculated by fitting the model by iteration. Standard errors of the parameters were computed by estimating the Hessian matrix after the iteration had terminated (Systat 5.2.1 software, Systat Inc.). Significance of the nonlinear regression equations was determined by comparing the calculated F-value and the distribution of Fisher-Snedecor (Statable Software). Significance of the difference between calculated C_s at different contaminating concentrations was tested by ANOVA using means and standard errors and by Tukey's multiple comparison test (Zar 1984).

Cadmium loss kinetics were described by assuming that the residual concentration at the end of the noncontaminating period could depart significantly from zero. Hence, the nonlinear regression model: $C_t = C_D + A\,e^{-t/l}$ was used, where C_D is the Cd concentration at the end of the noncontaminating period, A is the difference between concentration at the beginning and at the end of the noncontaminating period, l is a loss rate constant (d) (Kock & Kramer 1994). The parameters of the equations (C_D, A, l) were calculated by fitting the model by iteration (Systat 5.2.1 software, Systat Inc.). The level of significance was always set at $\alpha = 0.05$.

Results

Cadmium contamination of *Mytilus edulis*

For the contamination experiment through food, mussels were preliminarily contaminated in an aquarium by exposure to $Cd(NO_3)_2$ (20 µg Cd l^{-1} for 80 days). Mussels accumulated Cd linearly (linearity test: $p > 0.25$) at a rate of 1.53 ± 0.16 µg Cd g^{-1} dry wt d^{-1}. Three sets of contaminated mussels were successively provided to the asteroids. Cadmium concentration in the soft tissues of the mussels of the 2 first sets provided to the asteroids during the first 30 d of the contamination period was 109 ± 29 µg Cd g^{-1} dry wt. This concentration was found to remain unchanged in clean sea water (slope of the linear regression not significantly different from zero). Cadmium concentration in the last set of mussels did decrease significantly, but Cd loss was limited (23% maximum) and did not affect Cd uptake by the asteroids.

Cadmium contamination of *Asterias rubens*

Uptake and loss kinetics of Cd were followed in the pyloric caeca, the body wall and the skeleton of asteroids exposed to waterborne or dietary Cd (Fig. 1A - C). Regression models were calculated for each body compartment according to the exposure conditions. Different controls were performed to test possible fluctuations of Cd concentrations in the field or possible effects of the experimental devices. None of these controls revealed significant Cd variations in any asteroid compartments (slopes of the regression lines not significantly different from zero). Mean Cd concentrations in the controls are given in Table 1.

Actual concentrations of dissolved Cd in control containers used for contamination through water were regularly measured over a 12 h period. These measurements correspond to concentrations during the first 12 h of the exposure period and are a reasonable minimal estimate of the prevailing conditions during the whole period of exposure. After a rapid drop, the concentration in each container remained stable at about 0.5, 3, and 9 µg Cd l^{-1} (nominal concentration: 1, 5 and 20 µg Cd l^{-1}, respectively).

Cadmium uptake

Exposure to 5 and 20 µg dissolved Cd l^{-1} and to dietary Cd revealed significant uptake kinetics in the pyloric caeca. Kinetics were best described by the nonlinear model, and showed a saturation phase within 20 d (Table 2). Concentrations at saturation were compared by ANOVA and Tukey's multiple comparison test. They increased significantly with the contaminating concentration and were higher for contamination through food (Tables 2 & 3). Exposure to 1 µg

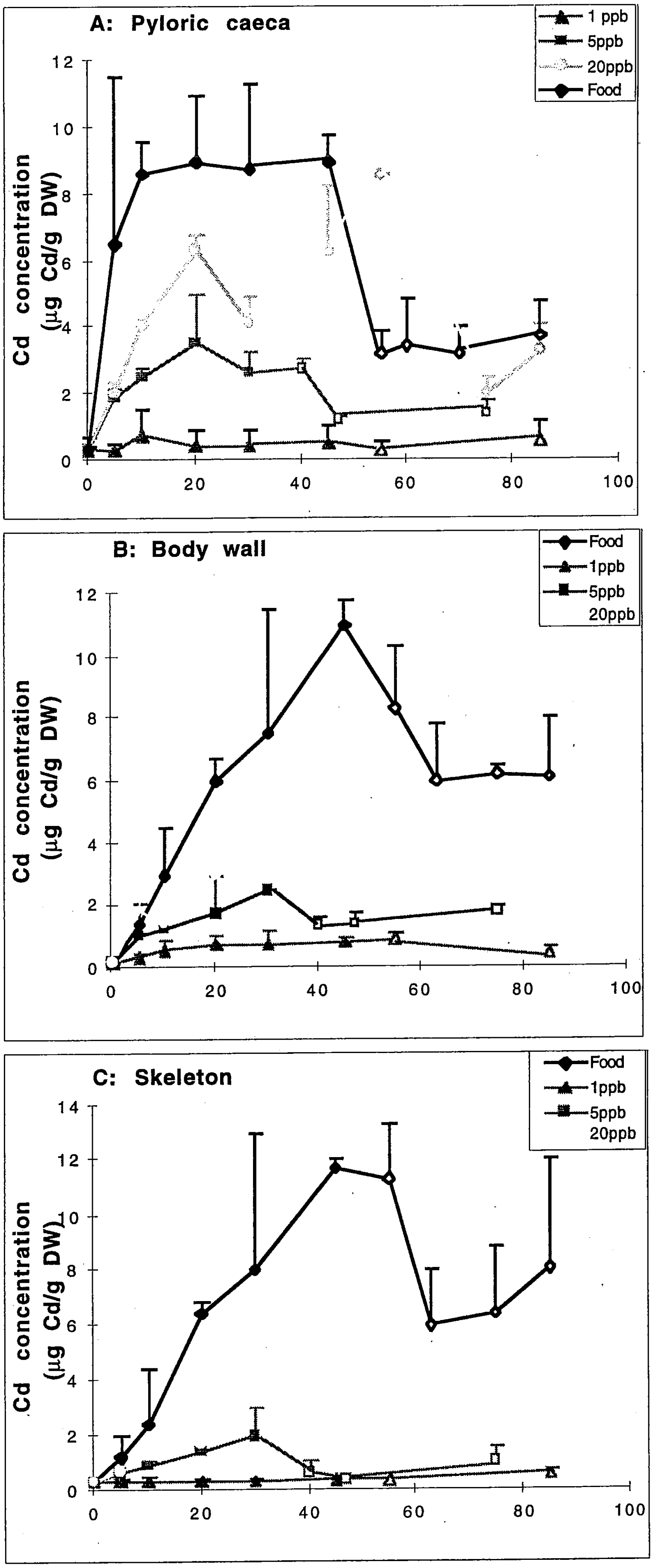

Figure 1: *Asterias rubens*. Uptake and loss kinetics of Cd (mean + SD when larger than dots) in 3 body compartments (pyloric caeca, body wall, skeleton) of asteroids exposed to Cd through food, or through sea water at a concentration of 1, 5, or 20 µg Cd l^{-1}. Solid and open symbols: uptake and loss kinetics, respectively.

dissolved Cd l^{-1} for 45 d did not result in a significant uptake of Cd in the pyloric caeca as tested by regression analyses (p = 0.17) (Table 2).

Exposure to 5 or 20 µg dissolved Cd l^{-1} or to dietary Cd revealed significant uptake kinetics in the body wall as well as in the skeleton. Kinetics were described accurately by linear regressions in both compartments (Table 2). Uptake rates (slopes) varied significantly as tested by ANCOVA. They increased significantly with the contaminating concentration as tested by Tukey's multiple comparison test, and were higher for contamination through food (Table 3). Comparisons between the uptake rate of the skeleton and that of the body wall at a same contaminating concentration (bilateral *t*-test) revealed that they were nonsignificantly different regardless of exposure mode, except at 1 µg Cd l^{-1}. Indeed, exposure to 1 µg dissolved Cd l^{-1} for 45 d did not result in a significant Cd uptake in the skeleton as tested by regression analyses (p = 0.57) (Table 2). On the contrary, uptake kinetics was significant at 1 µg Cd l^{-1} in the body wall and the hypothesis of linearity was rejected at this concentration. This kinetics showed a saturation phase (equilibrium reached within 30 d).

Cadmium loss

At the end of the Cd exposure, asteroids were transferred to uncontaminated regimens and the fate of Cd in the asteroids was studied (Fig. 1). Parameters of loss kinetics were calculated (Table 4). Cadmium concentrations in the body wall decreased rapidly, regardless of previous exposure mode. In contrast, loss of Cd by the skeleton was nonsignificant (regression analyses) regardless of previous exposure mode except in asteroids contaminated by waterborne Cd at the concentration of 20 µg Cd l^{-1}. Moreover, although mean Cd concentration did not increase significantly in the skeleton during exposure of asteroids to 1 µg Cd l^{-1}, the mean concentration at the end of the noncontaminating period increased significantly by 34%. At the end of the experiments, 83, 48, 72, and 77% of the total Cd load of the body wall was located in the skeleton of asteroids exposed to 1, 5, or 20 µg dissolved Cd l^{-1} or to dietary Cd respectively. By comparison, about 55% of the total body wall Cd load was located in the skeleton of control asteroids.

In the pyloric caeca of asteroids previously contaminated through food, Cd concentration decreased rapidly. On the other hand, the pyloric caeca of asteroids previously contaminated through water did not loose Cd during the first 10 d of the noncontaminating period. Afterwards, Cd loss was significant and rapid. Parameters of loss kinetics in the pyloric caeca of asteroids contaminated through water (Table 4) were hence calculated from the 10th day of the noncontaminating period.

Effect of Cadmium exposure on essential metal concentrations

Variations of Cu and Zn concentrations were measured all along the contaminating and noncontaminating periods in the same samples as Cd. Copper concentrations did not vary significantly (ANOVA, $\alpha=0.05$). Mean Zn concentration increased significantly and sharply in the pyloric caeca at the beginning of Cd exposures (42 to 134 %) regardless of exposure mode (Fig. 2). A return of Zn concentrations to basal levels was measured a few days later. Zn concentrations in the body wall and the skeleton were not significatively different as in controls (ANOVA, $\alpha=0.05$).

Discussion

Effective concentrations of dissolved Cd in the containers settled down after 8 h to about 0.5, 3, and 9 $\mu g\ Cd\ l^{-1}$, according to the nominal concentration. The differences from the nominal concentration could be attributed to interactions between Cd ions and some particulate and/or dissolved chemical species. Indeed, the placing of the container was followed by a resuspension of bottom muddy sediments and therefore of its interstitial waters. These waters are known to be anoxic and rich in sulfide (Fenchel & Riedl 1970). Hence Cd could have been rapidly adsorbed on resuspended particles and precipitated as sulfide species. The concentrations of nitrate injected with Cd were very low (maximum: 0.36 $\mu mol\ l^{-1}$) and were unlikely to have had any effect on primary production and hence on the generation of suspended particules. Indeed, background nitrate concentrations in the southern bight of the North Sea exceed 20 $\mu mol\ l^{-1}$ (NSTF 1993). After the initial drop, Cd concentrations remained stable. As Cd was renewed during the contamination experiment, adsorption and precipitation sites or reactives were progressively reduced and the effective concentrations in the containers became closer to the nominal concentrations. Hence, the measured concentrations of dissolved Cd probably correspond to the minimal bioavailable Cd concentrations in the containers. The lowest effective concentration of dissolved Cd (0.5 $\mu g\ Cd\ l^{-1}$) corresponds to highly polluted sea water, while the 2 other concentrations are encountered only very rarely in nature (Bryan 1984).

Mussels used in the present study accumulated Cd linearly during the preliminary contamination period up to a concentration of 110 $\mu g\ Cd\ g^{-1}$ dry wt. On return to uncontaminated sea water, Cd loss was either insignificant or limited. Continuous linear accumulation of Cd as well as low Cd loss over short periods are classically reported for mussels (Borchardt 1983, Bebianno & Langston 1991, Besten et al. 1991). Concentrations around 100 $\mu g\ Cd\ g^{-1}$ dry wt in the soft tissues of *Mytilus edulis* are rare in natural populations. However, concentrations exceeding this value have been reported in heavily contaminated areas (see Bryan 1984 for a review).

The experimental conditions of the present study were thus representative of acute contamination in nature. They had the advantage of allowing the tracking of Cd in the field. Furthermore, the concentration factor of Cd in an aquarium was demonstrated to be stable in *Asterias rubens* for ambient concentrations up to 3 µg Cd l^{-1} (Bjerregaard 1988). The results obtained with 2 of the effective Cd concentrations (0.5 and 3 µg Cd l^{-1}) used in the present study are thus probably representative of Cd processing by asteroids living in contaminating conditions.
Significant kinetics of Cd uptake in the pyloric caeca are best described by saturation models regardless of exposure mode. The steady-state concentration increased with the contaminating concentration. Concentration factors in the pyloric caeca calculated in the present study have been compared to values calculated by previous authors (Bjerregaard 1988, Besten et al. 1990) after Cd exposure in laboratory conditions (Table 5). They fit the range of values previously reported, therefore validating them in field conditions. In asteroids exposed to dietary Cd in the present study, mean concentration at saturation was 9 µg Cd g^{-1} dry wt. Similar concentrations at saturation have also been reported in the pyloric caeca of *Asterias rubens* exposed to 50 µg Cd l^{-1} (Besten et al. 1990) and 200 µg Cd l^{-1} (Bjerregaard 1988). A mean concentration of about 9 to 10 µg Cd g^{-1} dry wt would therefore be the maximal value possibly found in the pyloric caeca of asteroid populations exposed to a single Cd source. This would indicate a regulation of Cd concentration in pyloric caeca. According to Bjerregaard (1988), this behaviour is in sharp contrast with the large Cd uptake by many other marine invertebrates which may concentrate Cd up to several hundreds of µg g^{-1} dry wt. It is noteworthy, however, that under natural noncontaminating conditions, Cd bioaccumulation by *A. rubens* is not thoroughly regulated as Cd concentration in its body compartments increases as a power function of size (Temara et al. 1996).

According to Besten et al. (1990), the metallothionein-like proteins synthesized in the pyloric caeca of asteroids which have accumulated such high concentrations (9 to 10 µg Cd g^{-1} dry wt) do not reach their maximal saturation level. Moreover, Bjerregaard (1988) showed that concurrent exposure of *Asterias rubens* to selenite increases Cd uptake rates 5-fold without any saturation phase being reached. Hence it seems that, although they could accumulate more Cd, asteroids are endowed with an efficient protective mechanism that keeps the concentration of this metal relatively low. This has already been pointed out by several authors studying asteroid contamination by Cd or Hg (Pelletier & Laroque 1987, Bjerregaard 1988, Besten et al. 1989). However, it is noteworthy that Cd uptake by asteroids is proportional to the ambient concentration from 0.025 to 2.5 µg Cd l^{-1}, the concentration factor decreasing only at higher concentrations (Bjerregaard 1988). This makes *A. rubens* a valuable species in the study of Cd contamination. It provides a striking example of an organism controlling its internal Cd concentration under extreme conditions while reflecting environmental conditions in the usual range of Cd pollution. *A. rubens* may therefore be used as a suitable bioindicator of Cd

contamination as well as a model for a better understanding of Cd exclusion mechanisms. This exclusion could hypothetically be related to a translocation process from the pyloric caeca to other body compartments, to an excretory process that would increase metal elimination, or to other protective processes that would decrease metal bioavailability (e.g. increased mucus production). Warnau et al. (1995) showed that another echinoderm species, the echinoid *Paracentrotus lividus*, is protected against waterborne Cd by such mechanisms.
Cadmium translocation from the pyloric caeca to the body wall obviously occurred in asteroids contaminated through food. This translocation was still ongoing when the saturation phase had been reached in the pyloric caeca, as evidenced by the linear uptake kinetics shown by the body wall. Cadmium ingested with food is therefore rapidly distributed through the organism. Mercury is also rapidly translocated from the digestive system to the body wall in asteroids (Maheu & Pelletier 1994). Interestingly, Hg and Cd often share the same cellular ligants (Sorensen 1991). As saturation phases in the body wall are only observed after exposure to very high concentrations (4 w, 200 µg Cd l^{-1} ; Bjerregaard 1988), this body compartment can be considered as a virtually unlimited accumulation site for Cd in *Asterias rubens*.

In asteroids exposed to 1 µg Cd l^{-1}, the accumulation kinetics was significant and showed a saturation phase in the body wall, while no significant uptake was found in the skeleton under the same conditions. However, at the end of the 90 d experiment, a significant increase was observed in the skeleton, indicating that inclusion in this compartment is a slow process. Similarly, no significant accumulation was measured in the pyloric caeca at this contaminating concentration (nominal concentration: 1 µg Cd l^{-1}, measured concentration: 0.5 µg Cd l^{-1}). However, 0.5 µg l^{-1} of dissolved Cd is a high concentration hardly found in natural ecosystems, even at a polluted site, e.g. the polluted Western Scheldt (NSTF 1993). But Besten et al. (1991) showed that asteroids fed mussels from the polluted Western Scheldt in clean sea water accumulate Cd in concentrations up to 8 µg g^{-1} dry wt in the pyloric caeca. As the pyloric caeca uptake of waterborne Cd was nonsignificant at low concentration up to 45 d, Cd is accumulated in the pyloric caeca of asteroids living in polluted sites only because Cd had been previously bioconcentrated by its preys.

Loss kinetics studied in the present work were significant for all the studied body compartments except the skeleton. Thus, the retention time of Cd in the skeleton seems to be longer than in the other body compartments, even if asteroids exposed to 20 µg Cd l^{-1} did lose Cd during the noncontaminating period. Furthermore, asteroids exposed to the lowest studied concentration (1 µg Cd l^{-1}) accumulated Cd in the skeleton after noncontaminating conditions had been restored. After Cd exposure, the percentage of Cd in the body wall that was present in the skeleton was either close to or higher than in controls. It could thus be hypothesized that after the rapid Cd translocation from the digestive system to the body wall, Cd is slowly incorporated in the skeleton. Once in the skeleton, it is trapped for a large proportion and a long time. While internal organs and the soft part of the body wall would be protected by such a detoxification mechanism, biomineralization could be affected. Indeed, D'Andrea (1994) showed deleterious effects of Cd on skeletal regeneration in another echinoderm species, the ophiuroid *Microphiophilis gracillima.*

At the beginning of the exposure experiments, mean Zn concentration increased sharply in the pyloric caeca regardless of exposure mode. This increase of Zn concentration even occurred in the asteroids exposed to 1 µg Cd l^{-1}, although no significant Cd uptake was measured in those asteroids. This variation in Zn concentration could not be explained by a normal seasonal variation of essential elements as (1) controls were not affected during the same period and (2) exposures were actually de-synchronized (about 10 days). Neither it was an artifact due to Cd exposure. Indeed, the effect was measured in asteroids exposed to waterborne as well as dietary Cd, where external Zn bioavailability would have been different. Although the standard solution used for dosing asteroids did contain some Zn as a trace (maximal value certified by the manufacturer: 0.005%) the concentrations added to the water of the containers (0.05, 0.25 or 1 ng Zn l^{-1} when 1, 5 or 20 µg Cd l^{-1} were provided) are below natural background (Tappin et al. 1995), and the effect was about the same regardless of the amount of Zn provided. Although Zn concentrations were not measured in the contaminated mussels, the impact of a modified Zn concentration in the diet is unlikely. Indeed, according to Frazier and George (1983) there is no effect of Cd exposure on total soft-tissues Zn concentration in the bivalve *Ostrea edulis.* Moreover Cd decreases Zn accumulation in *Mytilus edulis* as well as in other mollusks (Simkiss & Mason 1984). It is therefore unlikely that mussels used as preys in our experiments were responsible for the induced variation in asteroids Zn concentration. Zinc could have been mobilized from other body compartments as the body wall. The effect of Cd uptake by asteroids on Zn concentration has also been studied by Besten et al. (1991). These authors measured Zn concentration at the end of the contamination period rather than along the entire kinetics. Exposure to 200 µg Cd l^{-1} during 5 weeks resulted in 30% decrease of mean Zn concentration in the pyloric caeca, while mean Zn concentration in the body wall was little affected. At the end of a longer exposure to Cd (25 µg Cd l^{-1}, 6 months), no significant change of mean Zn concentration was observed in exposed asteroids. Important effect of Cd exposure on Zn concentrations thus appears to be limited to the first days of Cd exposure. It is during this early phase of exposure that Cd toxic effects are the most important (Carpene et al. 1987, Besten et al. 1990). The latter ones are likely to decrease with exposure time as detoxification mechanisms are induced. It is noteworthy that the toxic effects of Cd are due to the fact that Cd^{++} and Zn^{++} ions are alike and compete for numerous cellular ligands (Sorensen 1991). A simultaneous increase of mean Zn concentration during Cd exposure could soften the deleterious effects of Cd either indirectly by competitive effect, or directly by the protective cellular properties of Zn (e.g.

limiting lipid peroxidation and DNA damage, Bettger & O'Dell 1981, Girotti et al. 1985). It could also trigger the synthesis of metallothioneins.

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Uptake and fate of lead in the common asteroid *Asterias rubens* (Echinodermata)

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Abstract. Lead uptake kinetics in the body compartments of *Asterias rubens* individuals exposed through sea water to different concentrations (5, 20, 50 µg Pb l^{-1}) were studied during 30 days. Uptake kinetics were always best described by linear regressions. In every compartment, uptake rate increased significantly with the contaminating concentration. At a given concentration, the uptake rate of the central digestive tract was always significantly higher than that of the other compartments. After short-term exposures (50 µg Pb l^{-1}, 8 days), fate of accumulated Pb was followed in asteroids kept in non-contaminated water during 20 days. The central digestive tract and the pyloric caeca lost Pb according to an inverse exponential and concentrations returned to initial values after 4 - 8 days. Pb concentrations in the body wall decreased linearly. Pb concentrations in the skeleton did not vary significantly. In the gonads, Pb concentrations kept on rising while exogenous Pb was no more supplied. Pb loss from the body wall and the digestive compartments could account for such endogenous source to the gonads. It is suggested that Pb accumulated in the gonads is expulsed later through spawning. According to the parameters of uptake and loss kinetics, the central digestive tract and the skeleton are proposed as complementary temporal bioindicators of Pb contamination.

Key-words: *Asterias rubens*, Pb exposure, uptake kinetics, loss kinetics

1. Introduction

World production of lead (Pb) has continuously increased since its early use by ancient civilizations (Settle and Patterson 1980). According to Smith and Flegal (1995), 50% of the Pb bulk produced during the three last milleniums (over 300 millions tons) has been discharged to the environment as a contaminant. Pb is a particle-reactive element (Balls 1989). Its dispersion is hence rapid and wide, leading to its accumulation in many environmental matrices. That is illustrated by the temporal changes in Pb concentrations in Greenland snows (Boutron *et al* 1991) or by the presence of anthropogenic Pb in deep-sea sediments (Veron *et al* 1987). Although severe regulations of main industrialized countries have reduced the consumption of Pb in some materials (particularly in gasoline), global emissions are not showing a significant downwards trend (Smith and Flegal 1995). Therefore Pb will still cause problems because of its conservative status and its increasing use in some parts of the world.

The North Sea receives high quantities of Pb (up to 11.10^6 tons/year, Kersten *et al* 1988) which results in high environmental levels (dissolved Pb: up to 0.23 μg Pb l^{-1}; particulate Pb: up to 844 μg Pb g^{-1} DW, Tappin *et al* 1995; Pb in the fine granulometric fraction of the sediment: up to 350 μg g^{-1}, Kersten and Klatt 1988). As a particule-reactive metal, Pb is readily incorporated in food webs based on filter-feeders *via* the suspended particulate matter.

As an approach to tackle the study of the transport mode and the fate of that element in estuarine and coastal benthic organisms, the present work was undertaken to study Pb fluxes in the common asteroid *Asterias rubens,* L, a predator preying on filter-feeders. The purpose of this study was to quantify Pb uptake and loss of the main body compartments of the asteroid, which will check the relevance of proposing *A. rubens* as a suitable bioindicator of Pb contamination in North Sea littoral environments.

2. Materials and methods

2. 1. COLLECTION AND EXPOSURE OF INDIVIDUALS

Individuals of *Asterias rubens* Linneaus, 1758, were collected in the Eastern Scheldt (Scharendijke, The Netherlands) by SCUBA divers. They were exposed in aquariums to Pb as $Pb(NO_3)_2$ (Titrisol, certified quality, Merck). Sea water (Salinity: 29‰) had been pumped in the Eastern Scheldt (Scharendijke) and filtered (plankton net; mesh size: 50 μm). Asteroids were fed *ad libitum* uncontaminated mussels (*Mytilus edulis,* L) from the same site. Water, Pb content, and preys were daily renewed.

To assess Pb uptake kinetics of the asteroid body compartments, three sets of asteroids collected in June 1994 (postspawning period) (n = 18 in each set; size: 6 ≤ large ray ≤ 7 cm) were exposed for 30 days to 3 different Pb concentrations in sea water (nominal concentrations: 5, 20 and 50 µg Pb l^{-1}; temperature: 15 ± 0.5 °C). Three asteroids were sampled in each set at the beginning of the contamination period (day 0), then the 1st, 5th, 10th, 20th and 30th day.

To assess the fate of Pb in the asteroid body, loss kinetics of the body compartments were studied in asteroids collected in December 1994 (gametogenesis period). Asteroids were exposed to 50 µg Pb l^{-1} during 8 days and then placed in sea water without added Pb for 20 days. Water and mussels were daily renewed. Pb concentrations were measured in the body compartments of 4 asteroids sampled the 9th, 12th, 16th, 21th, 25th, 28th days.

In order to take into account individual fluctuations of Pb concentrations under non contaminating conditions and to assess possible effects of the experimental device, 18 asteroids were placed for 30 days in an aquarium and submitted to the same treatment as the experimental asteroids except for Pb exposure (control experiment). Samples were regularly collected and Pb concentrations were measured.

2.2. SAMPLE PREPARATION AND METAL ANALYSIS

Each sampled asteroid was dissected just after collection and the body compartments were separated. Sample processing was performed as previously described (Warnau *et al* 1995). Pb concentrations were measured by atomic absorption spectrometry (GBC 906 AA) after acid digestion of the samples. The reproductibility of the methodology was assessed using an internal standard (homogenized powder of asteroid body wall). Pb concentrations of 3 internal standards were analysed with each sample set. The variations of the measurements (n=15) were low (always in between mean ± 2 SD of the first sample value) all along the analyses. A certified material (*Mytilus edulis*, CRM n° 278, Community Bureau of Reference; Certified Pb concentrations = 1.91 ± 0.04 µg g^{-1} DW) was analysed along with the experimental samples to assess data quality (measured Pb concentration of the certified material = 1.9 - 2.0 µg g^{-1} DW - min and max; n = 5). Detection limit (mean + 3 SD of the blank) equalled 14 µg Pb l^{-1}. Measurements below detection limit were replaced by half of this value (Black 1991).

2.3. DATA ANALYSIS

Uptake kinetics of the asteroid body compartments were fitted using simple linear regressions. Linearity was tested by the linearity test for regression with replication (Zar 1984). Significance of the slopes of the linear regression equations was tested by 1-way analysis of variance (ANOVA). Comparisons between slopes of the linear regression equations were tested by analysis of covariance (ANCOVA) followed by Tukey's multiple comparison test (multiple comparisons between more than 2 regression slopes) (Zar 1984).

When significant, loss kinetics were described either by simple linear regression or by the nonlinear regression model: $C_t=C_D + A^* e^{-kt}$ where C_D is the Pb concentration at the end of the decontamination period, A is the difference between mean concentration at the beginning and at the end of the decontamination, k is the loss rate constant (dimension: d^{-1}) (Kock and Kramer 1994). When mean concentration increased during depuration, kinetics was described using the nonlinear model: $C_t-C_0 = (C_s-C_0)\ (1-e^{-kt})$; where C_0, C_t, and C_s are the Pb concentration at the times 0, t and steady-state; k is the uptake rate constant (dimension: d^{-1}) (adapted from Whicker and Schultz 1982). Parameters of the equations (C_0, C_s, k) were calculated by fitting the model by iterations (Systat 5.2.1 software, Systat Inc.). Standard errors of the parameters were computed by estimating the Hessian matrix after iterations have terminated (Systat 5.2.1 software, Systat Inc.). Significance of the nonlinear regression equations was determined by comparing the calculated F value and the distribution of Fisher-Snedecor (Statable Software). The level of significance was always set at $\alpha= 0.05$.

3. Results

Regression models were calculated for each body compartment according to the exposure mode. Fluctuation of Pb concentration in all compartments of control organisms was nonsignificant (slope of the regression line not significantly different from 0).

The results obtained during experimental contaminations show that along the selected period (30 days), the uptake kinetics are linear and that asteroids are able to concentrate the contaminant up to high levels in every body compartment (Table 1). After 1 month of exposure to 50 µg Pb l^{-1}, tissular concentrations reached 72 ± 23 µg Pb g^{-1} in the central digestive tract, 43 ± 10 µg Pb g^{-1} in the skeleton, 36 ± 10 µg Pb g^{-1} in the body wall, and 32 ± 5 µg Pb g^{-1} in the pyloric caeca. In every compartment, the uptake rate increased significantly with increasing contaminating concentration as tested by analysis of covariance and slope multiple comparison test. At any contaminating concentration, the uptake rate of the central digestive tract was significantly higher than that of the other compartments (Table 1).

The fate of Pb in the asteroid body compartments was assessed when noncontaminating conditions had been restored (Fig. 1). Parameters of equations of the regression models describing variations of Pb concentration during such period are given in Table 2. Concentration in the pyloric caeca and in the central digestive tract decreased exponentially. During the studied noncontaminating period, the central digestive tract and the pyloric caeca lost on average a Pb load of 1.3 µg and 18.8 µg, repectively. Loss of Pb by the body wall was significant and linear, but slower. On average, it lost 133 µg Pb. On the contrary, loss of Pb by the skeleton was nonsignificant (p ANOVA = 0.16). Pb concentrations in the gonads kept on rising significantly and sharply, although exogenous Pb source was stopped. During the first 4 days, the gonads took up on average 21 µg Pb; equilibrium was reached afterwards.

4. Discussion

To our knowledge, this is the first report of Pb uptake and loss kinetics in echinoderms. Uptake kinetics of Pb by the body compartments of *Asterias rubens* were linear all through the studied contamination period. Equilibrium states were not reached at any contaminating concentration. However, in every body compartment, the uptake rate increased significantly with the contaminating concentration. It can therefore be suggested that Pb uptake is a dose-dependent mechanism in *Asterias rubens*. Regardless of exposure concentration, the uptake rate of the central digestive tract was always significantly higher than that of the other body compartments.

When noncontaminating conditions were restored, there was a rapid and total elimination of the accumulated Pb from components of the digestive system (the central digestive tract and the pyloric caeca). Simultaneously, Pb concentration in the gonads was keeping on rising while no more exogenous Pb source was available. Interrestingly, the total amount of Pb lost by the digestive compartments averaged the amount of Pb taken up by the gonads (20 µg Pb). In *A. rubens*, a nutrient translocation occurs from the digestive and storage organs (the central digestive tract and the pyloric caeca, respectively) to the gonads during gametogenesis (Voogt *et al* 1985). We suggest that Pb follows this transport of nutrients. Pb could then be stored in the growing gonads before its elimination through spawning. Although the contaminant could be toxic to the developing gametes, Dinnel *et al* (1989) and Warnau and Pagano (1994) have shown that Pb has no harmful effects on mature echinoid gametes even at concentrations much higher than those found in the marine environment. This form of contaminant elimination would probably not be a general mechanism in *A. rubens*. It is restricted to the period of gonad growth and does not occur for all metals. For instance, Cd accumulation in the gonads is very low, even in heavily contaminating conditions and other forms of detoxification are then involved (Besten *et al* 1990).

This translocation from the digestive system to the gonads could partly account for the allometric relationships recorded in the digestive system of *A. rubens* (Temara *et al* 1996). In this compartment, Pb concentrations are inversely related to asteroid size. It was suggested that this allometric relationship is related to the achievement of sexual maturity, when part of accumulated Pb would directed from the digetive system to the gonads (Temara *et al* 1996). The present results strongly sustain this hypothesis.

Mean Pb concentration in the skeleton did not vary significantly after return to noncontaminating conditions. Pb is known as a bone-seeking element, regardless of skeleton nature (Sturesson 1976, Shen and Boyle 1988, Beeby and Richmond 1989, Hu *et al* 1995, Temara *et al* 1995, 1996). In mammals, Pb has a half-life of years to decades in bone (Steenhout 1982, Chritofferson *et al* 1987). In asteroids, Pb seems also to be thightly bound to the skeleton.

This study enables us to propose the body compartments of *A. rubens* as complementary bioindicators of Pb contamination. The low concentrations of Pb in the central digestive tract under natural noncontaminating conditions (Temara *et al* 1993, Temara *et al* 1996), and its high uptake and elimination rates (present study), make this compartment a valuable short-term bioindicator of Pb contamination. On the contrary the skeleton, which continuously grows and is apparently a trap for Pb, will be a valuable long-term bioindicator. It is suggested to sample asteroids belonging to the largest size-class in order to avoid allometric effects (Temara *et al* 1996).

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Kinetics of lead uptake by the skeleton of the asteroid *Asterias rubens* (Echinodermata)

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Abstract

Capacity of Pb uptake from sea water (5, 20, 50 μg Pb l^{-1}) by the skeleton of the asteroid *Asterias rubens* was measured *in vitro* during 72 h. Uptake kinetics may be resolved into short-term and long-term components. The short-term components are described by a linear model while the long-term components are described by a nonlinear model. Kinetics began with a steep uptake and reached a saturation phase within the first 24 h. Concentration at saturation depended on the Pb concentration in the solution. Uptake by the skeleton ranged from 44 to 57 % of the total dissolved Pb in solution. Comparison with uptake models established for other metals on pure calcite suggests that Pb taken up by the asteroid skeleton is adsorbed. The measured adsorption could account for a significant proportion of *in vivo* Pb accumulation.

Key words: *Asterias rubens* · Lead - Skeleton · Uptake kinetics · Adsorption

1 INTRODUCTION

Lead is readily accumulated in carbonate skeletons of many organisms (see e.g., Sturesson 1976, Shen and Boyle 1988, Beeby and Richmond 1989). Interestingly, this accumulation occurs in both aragonite and calcite skeletons, although incorporation of Pb should theoritically be favoured in the aragonite lattice (Speer 1983). In aragonite skeletons of scleractinian corals, Shen and Boyle (1988) presented evidence that Pb is indeed incorporated in the crystal lattice. As far as we know, no information is available about the location of Pb in calcite skeletons. Hypotheses about Pb location in calcite skeletons include incorporation in the crystal lattice (as suggested by Sturesson, 1976), adsorption on the biomineral surface, and complexation with the intraskeletal organic matrix.

The skeleton of echinoderms is made of high-magnesium calcite. In asteroids, ophiuroids, crinoids, and echinoids the skeleton represents a high proportion of the body wall which generally shows high Pb concentrations (4.5 to 30 μg Pb g^{-1} DW according to the studied species) (Krönke 1987, Kersten and Krönke 1991, Gounin 1992, Lawrence *et al.* 1993, Temara *et al.* 1993, Warnau *et al. in press*). Moreover Temara *et al.* (1993) showed that in *Asterias rubens*, the major part of the total Pb body load is located within the skeleton.

The aim of the present study is to assess the adsorption capacity of the echinoderm skeleton towards lead.

2 MATERIAL AND METHODS

2.1 *Collection of samples*

Individuals of *Asterias rubens* Linneaus, 1758, were collected in the eastern Scheldt (Scharendijke, The Netherlands) by SCUBA divers. Ossicles were isolated with 1 ‰ Proteinase N (Serva) (Dubois and Jangoux 1985) and cleaned . Subsamples were observed by scanning electron microscopy (ISI-DS 130 SEM) to check the absence of etching and the complete cleaning of the ossicles. Ossicles were autoclaved (110°C, 20 min) prior to use and manipulations were conducted in sterile conditions.

2.2 *Sample preparation and metal analysis*

The sea water used in the experiments was first equilibrated with the asteroid skeleton. For that, samples (n = 120; 1 g each) of autoclaved ossicles were placed each in 110 ml artificial sea water

(30 g l^{-1}, Sea salts Sigma) in polyethylene containers (Vol. : 125 ml). Open containers were then placed in an incubator (15 ± 0.5 °C) during 8 h so that carbonate concentration in the sea water reached equilibrium with the ossicles. Filtered (Sterivex, Millipore 0.22 µm) fresh air was permanently supplied in the incubator. Subsequently, the water was collected and the ossicles were discarded. Equilibrated water was shared out between 120 clean polyethylene containers (100 ml each) and a precisely known quantity of autoclaved ossicles (approximately 0.55 g) was placed in each container. The containers were then kept open for 24 h in the incubator. Afterwards, Pb (as Pb NO_3, Titrisol, certified quality, Merck) was added at a concentration of 5, 20, or 50 µg Pb l^{-1}(30 containers at each concentration). A set of controls did not receive Pb. Containers were closed, placed in the incubator, and shaken automatically.

The pH (precision: 1 unity/100, Radiometer 26 pH meter, Copenhagen) was measured in each container at the start and at the end of the experiments. All through the experiments, pH variations were very low (within 1/10 pH unity). After 1 min, 3 min, 5 min, 10 min, 30 min, 1 h, 6 h, 25 h, and 72 h, the Pb solution was removed from 3 containers at each Pb concentration, the ossicles were rinsed with milliQ water (Millipore) and dried (85°C, 48 h). They were then processed for Pb analysis by atomic emission spectrometry (ICPS-Jobin Yvon 38+) as described by Warnau *et al.* (1995).

2.3 *Data analyses*

Uptake kinetics during the first 30 minutes were described by linear regressions according to Zar (1984). Subsequent kinetics (1-72 h) were described by the nonlinear regression model: $C_t - C_0 = (C_s - C_0)(1 - e^{-kt})$; where C_t is the Pb concentration in the ossicles at the time t, C_0 is the initial concentration, C_s is the concentration at saturation, and k is the uptake rate constant (dimension: h^{-1}) (adapted from Whicker & Schultz 1982). The parameters of the equation (C_0, C_s, k) were calculated by fitting the model by iterations (Systat 5.2.1 software, Systat Inc.). A certified material (*Mytilus edulis*, CRM n° 278, Community Bureau of Reference; Certified Pb concentrations = 1.91 ± 0.04 µg g^{-1} DW) was analysed along with the experimental samples to assess data quality (measured Pb concentration of the certified material = 1.91 - 1.95 µg g^{-1} DW - min and max; n = 5). Detection limit (mean + 3 SD of the blank) equalled 14 µg Pb l^{-1}.

3 RESULTS

Kinetics of *in vitro* Pb uptake by *Asterias rubens* ossicles are presented in Fig. 1. Control samples incubated in sea water without added Pb showed no significant variation of their Pb concentrations (parameters of the linear regression: $R^2 = 0.001$, p=0.6). Data from short-term (first 30 minutes) and long-term (1-72 h) components of the uptake kinetics were analysed separately as they presented different trends. During the first 30 minutes of the experiments (Fig. 1A), no significant uptake was measured on the ossicles submitted to the lowest contaminating concentration (5 µg Pb l^{-1}) (parameters of the linear regression: R^2= 0.001, p=0.8). Significant uptake occurred in samples submitted to 20 and 50 µg Pb l^{-1}(parameters of the linear regressions: R^2= 0.27, slope= 0.02, p=0.05; R^2= 0.70, slope= 0.04, p<0.01, respectively). The mean uptake rates were thus 1.18 and 2.64 µg Pb g^{-1} DW $hour^{-1}$, respectively. The long-term components of the uptake kinetics showed a saturation phase that was reached within the first day (Fig. 1B). Concentration at saturation increased with increasing contaminating concentration (Table 1). The fraction of the total dissolved Pb in the sea water that was taken up by the ossicles varied from 44 to 57 % (Table 1).

4 DISCUSSION

Uptake of Pb by the skeleton of *Asterias rubens* began by a rapid phase that took place during the very first hours of the experiments, and the kinetics eventually reached a saturation phase between 6 and 25 h, whatever the studied contaminating concentration.

Studies on the uptake of other divalent cations (X^{++} = Cu^{++}, Mn^{++}, or Cd^{++}) from sea water solutions, spiked with $CaCO_3$, onto calcite showed that there is an initial (first 24 h) rapid uptake (interpreted as an adsorption stage) followed by a steady removal of the metal from the solution (interpreted as a crystallization stage of $X_xCa_{1-x}CO_3$) (Morse 1986). No such data are available for Pb. However, Pb was shown to be one of the most effective inhibitors of calcite dissolution/precipitation kinetics indicating that Pb is very probably adsorbed on calcite (Morse 1986). Thus, the rapid increase of Pb concentration in the asteroid ossicles during the first hours of the present experiments should correspond to the adsorption of the ion on the skeleton. As the sea water used in the present experiments was not spiked with $CaCO_3$, no further $PbCO_3$ (or $Pb_xCa_{1-x}CO_3$) crystallization could occur. A further increase of Pb concentraton in the ossicles would be dependent on the growth of the asteroid skeleton and hence could only take place in living asteroids. The present results indicate that a significant adsorption of Pb can occur on asteroid ossicles and that this mechanism could account for at least a part of Pb accumulation in the echinoderm skeleton.

The adsorption of Pb on echinoderm ossicles raises the question of the possible impact of this metal on echinoderm skeleton formation. Although Pb was shown to have no effect on shell growth in molluscs (Strömgren 1982), it does induce skeletal anomalies in echinoid larvae (Warnau and Pagano 1994). Though the latter effect could be totally indirect, it emphasizes the need to further investigate Pb effects on biomineralization in echinoderms, especially as this metal is a major threat in polluted coastal environments (North Sea Task Force 1993).

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Quantification of metallothioneins in the common asteroid *Asterias rubens* (Echinodermata) exposed experimentally or naturally to cadmium

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Abstract: Uptake and intracellular fate of Cd was assessed in pyloric caeca of the common asteroid *Asterias rubens* exposed experimentally or naturally to Cd. Cd partitioning among intracellular metal-binding pools was studied by graphite furnace atomic absorption spectrometry of heat-treated cytosolic fractions which had been separated by gel filtration chromatography. Low molecular mass proteins bound >80% of cytosolic Cd and appeared to become saturated with the metal after experimental exposure to 20 μg Cd l^{-1} for 10-20 days. These proteins presented several features of metallothioneins (MTs), namely molecular mass (13-22 kDa), heat-stability (90°C, 10 min), high Cd content, and high thiolic content as determined by differential pulse polarography. Analysis of chromatographic fractions indicated that the majority of thiolic groups (66-73%) present in heat-treated cytosol was associated with the MT pool. Subsequent determinations of MT concentrations were made using whole cytosol extracts after correcting for interference from thiolic groups present in residual, heat-stable, high molecular mass proteins. Asteroids collected from unpolluted sites in SW England, SW Netherlands and SW Norway contained basal levels of 2.5-4.5 mg MT g^{-1} dw. Asteroids experimentally exposed to Cd (20 μg Cd l^{-1}) responded by a 2-fold increase in the production of MT after 30 days. Turnover of MTs was rapid in *A. rubens* and probably accounted for the low net increase in protein and characteristic rapid loss of Cd from the pyloric caeca. Alkaline phosphatase (AP) activity was not significantly affected during Cd exposure in the laboratory. Asteroids collected in the heavy metal polluted Sørfjord (SW Norway) contained significantly higher MT concentrations (5-5.6 mg MT g^{-1} dw) and significantly lower AP activity in the most heavily contaminated region, though these two parameters were not correlated.

Key words: Metallothionein-*Asterias rubens* - Differential pulse polarography - Heavy metals

Introduction

Heavy metals of classes Ib and IIb (Mendeleev classification) are of most concern for ecotoxicologists. Some of these metals (Cu and Zn for example) are essential to life, whilst no biological function has been reported for others (Cd, Hg, Ag), though all produce toxic effects when taken up in excess (Sorensen 1991, Clark 1992). Because of their analogous electroaffinities, elements belonging to the same class share identical preferences for intracellular ligands when ionized (Brezonik et al. 1991). Class Ib and IIb elements, in particular, have a high affinity for peptidic sulphydryl groups (Viarengo & Nott 1993). In this context, Cu and Zn are used biologically as co-factors in a vast number of enzymes (Sorensen 1991), but they may be substituted by Cd, Hg or Ag in contaminated, impaired systems. Other intracellular ligands include very low molecular mass peptides (VLMMP) such as glutathione (Wofford & Thomas 1984) or low molecular mass proteins (LMMP) such as metallothioneins (MTs) (Roesijadi 1992). Zn and Cu bind to constitutive MTs and are released according to cellular demand in a process of homeostasis (Kägi 1991). Cd, Hg and Ag also bind to MTs in a process of detoxification. When the system is overloaded, *de novo* synthesis of MT may occur which may remove potentially toxic free metal ions from the cytoplasm (Olafson et al. 1979).

Based on the differential pulse polarographic (dpp) estimation of thiolic proteins first described by Brdicka et al. (1933), Thompson & Cosson (1984) updated a sensitive method for the quantification of MTs in marine organisms. This technique has been applied to crustaceans (Olafson et al. 1979) and to molluscs (Bebianno & Langston 1991, Pavicic et al. 1993), but to our knowledge little quantitative information is available for most other marine invertebrates, particularly for a widely distributed and abundant phylum: Echinoderms.

Occurence of MTs has been demonstrated in echinoids (spermatozoa and premetamorphic stages) (Nemer et al. 1985, De Prisco et al. 1991). LMM metal-binding proteins have previously been reported in the pyloric caeca of *Asterias rubens* (Besten et al. 1990) but were not demonstrated to be MTs and their quantification has still not been attempted. Interestingly, the pyloric caeca have been identified as the main Cd accumulating body compartment in the species (Temara et al. 1993). It is worty noting that *A. rubens* is a major benthic predator in the North-East Atlantic (Hayward & Ryland 1990) and is abundant along the coast, a feature which promotes its potential use in biomonitoring. The present study aims to quantify MT-like proteins in pyloric caeca of *A. rubens* exposed naturally or experimentally to Cd and to assess the relation between MT concentrations and the degree of impairment of the functional state of the asteroid assessed through alkaline phosphatase activity.

Materials & Methods

Metallothionein concentration, cytosolic distribution of thiols and metals (Cd, Zn and Cu), and alkaline phosphatase activity were measured in pyloric caeca of *Asterias rubens* (Linnaeus, 1758) exposed to Cd in the field or in aquaria. The size of individuals collected was 6 to 9 cm, measured from the tip of the longest arm to the opposite inter-radius.

Description of the samples

(i) *Experimentally exposed asteroids*. Asteroids were exposed for 45 days in the field to waterborne Cd. Contamination *via* sea water was performed in plexiglass containers into which Cd was injected (nominal concentration: 20 μg Cd l^{-1}). The containers (each containing 28 asteroids) were anchored in the sediment at a depth of 9 meters in the Eastern Scheldt (The Netherlands). Asteroids were fed non contaminated mussels (1 mussel $asteroid^{-1}day^{-1}$). Mussels, water and Cd were renewed twice a day. Control asteroids were treated identically to exposed individuals except for Cd administration. Samples were daily collected by SCUBA divers. After exposure, contaminated and control asteroids were placed in polyethylene meshed cages, and fed non-contaminated mussels for 40 days. Cadmium uptake, and subsequent loss kinetics, in different body compartments of the asteroids are described elsewhere, together with a full description of the sampling procedure (Temara et al. in press). Subsamples of pyloric caeca were collected and frozen (-20°C for 1 week, then -80°C for 12 months) for MT and subcellular metal distribution analysis.

In order to validate the methodology, particularly the effect of freezing on MT content, asteroids from an uncontaminated site (Bigbury, Devon, UK) were exposed in laboratory aquaria to 1, 5, 20 or 200 μg Cd l^{-1} for up to 3 weeks. They were provided with non contaminated mussels every day. Water and Cd in the aquaria were renewed every other day. Fresh samples of pyloric caeca were immediately processed and analysed, following dissection, thereby avoiding a freezing stage.

(ii) *Asteroids from contaminated field sites*. Specimens of *A. rubens* were collected in August 1995 along a fjord in South-West Norway (Sørfjord) which has two ore smelters at its head (Fig 1). The location is an exceptionally contaminated area, where discharges (Cd, Zn, Cu, Hg, Pb) from metallurgical industry have occurred for more than 65 years (Skei 1981, 1993). Asteroids were collected at sites close to each smelter (sites S1 and S2), 12 km further downstream (site S3) and outside of the contaminated fjord (site S4). Figure 1 locates sampling sites, total Cd concentrations measured in pyloric caeca of sampled asteroids can be found in the caption. During sample processing, subsamples of pyloric caeca were collected and frozen (-20°C for 1 week, then -80°C for 4 months) for MT and subcellular metal distribution analysis.

Preparation of the samples

Pyloric caeca from each individual (usually three at each sampling) were subdivided in 3 subsamples. They were weighed (wet weight) and homogenized (Ultra-turrax) on ice with either 4 volumes of TMS buffer (subsamples 1, for enzymatic analysis, see below) or 4 volumes of 0.02 M Tris-HCl buffer (pH 8.6) (subsamples 2) for MT determination. Subsamples 3 from the three animals of a given sampling were pooled and homogenized in identical Tris-HCl buffer for subsequent gel-filtration chromatographic characterisation of cytosolic metals, thiolic groups and UV-absorption properties, following methods described fully elsewhere (Langston & Zhou 1986, Bebianno & Langston 1995). Briefly, for MT and metal characterisation, an aliquot (2ml) of subsamples 2 and 3 was centrifuged at 30,000 g for 1h at 4°C. The supernatant (cytosol) was separated from the pellet, heated (90°C, 10 min) to precipitate the majority of proteins (putatively leaving only heat-stable MT in solution) and subsequently centrifuged at 30,000 g for 90 min at 4°C. This heat-treated cytosol was then analysed polarographically for MT (subsamples 2) or chromatographed (subsamples 3) for identification of the major metal-binding and thiol-containing pools. A further aliquot (3ml) of each homogenate was taken and dried at 85°C for 48h for wet:dry weight ratio determination in order to express data on a dry weight basis.

Heat treatment of an aliquot of cytosol from one of the pooled samples (asteroids exposed to 200 µg Cd l^{-1} for 21 days) was omitted so that the metal-binding/-SH contribution of heat-denaturable ligands, principally in the high molecular mass (HMM) protein pool, could be assessed.

To test for MT polymerisation, some samples were homogenized in 0.02 M Tris-HCl buffer (pH 8.6) containing 2mM dithiothreitol (DTT), an antioxidant that prevents polymerization (see, for example, Pavicic et al. 1993) and analysed as described for the others.

Chromatographic separation of metal-containing pools

The procedure and conditions used for separation of metal-binding components in asteroids were modified from Langston & Zhou (1986). Aliquots of heat-treated cytosol (0.5 ml) of subsamples 3 were chromatographed on a Sephadex G-75 column (1.5 x 60 cm) and eluted with 0.02 M Tris-HCl buffer (pH 8.6) at 4°C. A flow rate of 0.2 ml min^{-1} was used to elute the samples which were collected as 2-ml fractions. The UV-absorbance of the fractions was measured at 254 and 280 nm (Varian Cary 1 UV-Visible Spectrophotometer).

Thiolic content of chromatographed fractions (individual or pooled) was measured by differential pulse polarography (dpp), described below. Cadmium, Zn and Cu concentrations in these fractions were measured by graphite-furnace atomic absorption spectrometry (Varian AA-300) using Zeeman background correction.

The column was calibrated for apparent molecular mass estimations with bovine serum albumin, cytochrome C, cobalt chloride and rabbit MT-I as standard markers. The first three were detected by spectrophotometry at 290, 418 and 280 nm, respectively; rabbit MT-I was detected by dpp. The linear equation which best describes the relationship between molecular mass (MM) and fraction number was:

$$\text{MM (kDa)} = -61 \ln(\text{fraction number}) + 210 \qquad R^2: 0.96,\ p<0.001 \qquad [\text{Eq. 1}]$$

Quantification of cysteine-rich proteins

A dpp assay for -SH compounds, based on the Brdicka reaction (Brdicka 1933), was used in the determination of MT in whole cytosol extracts and the thiolic response in chromatographic fractions from selected samples. The chromatographic separation is necessary to assess the relative dpp response generated respectively by the MT pool and the potentially interfering thiols associated with HMM proteins. Heat treatment followed by high speed centrifugation would be expected to denature and remove the latter from cytosolic samples as observed in *Mytilus edulis* and *M. galloprovincialis* (Bebianno & Langston 1991, 1992). However the tissues of some species, for example digestive gland of *Littorina littorea* (Langston et al. 1989), contain heat-stable thiolic HMM proteins which can lead to over-estimation of the MT content of whole cytosol preparations, unless corrected.

Polarographic measurements were made with a PARC Model 174A analyser, a PARC/EG&G Model 303 static mercury drop electrode and a flat-bed X-Y recorder. The three-electrode system consisted of a bevelled capillary mercury working electrode, a platinum counter electrode and a Ag/AgCl reference electrode. The Brdicka supporting electrolyte contained 1M NH_4Cl, 1 M NH_4OH and 2mM $[Co(NH_3)_6]Cl_3$. The electrolyte was prepared every 4 days and stored at 4°C when not in use.

Ten millilitres of electrolyte were dispensed to the polarographic cell, together with 100 µl Triton X-100 (0.0125 %, v:v) and 10 µl of heat-treated cytosol (diluted 5 times in 0.02 M Tris-HCl buffer, pH 8.6). Samples of 250 µl were used for analysis of individual chromatographic fractions. The solution was then purged for 2 min with purified N_2 prior to analysis. Scanning was from -1.4V to -1.6V at 2mV s^{-1}. Modulation amplitude was 50mV and drop time 1 s.

In the absence of an echinoderm MT standard, quantification of MT in the cytosol was based on rabbit liver MT-I (working standard 10 mg l^{-1} in distilled water), using the method of standard additions (Bebianno & Langston 1989). Concentrations of MT in the whole cytosol were determined as mg per g dry weight of tissue, taking into account the relative proportions of the polarographic response attributable to MT and non-thionein fractions (*see above*). Thiolic content of individual chromatographic fractions from selected samples, expressed as a function of polarographic response (µA), was corrected to the original dry tissue weight (µA g^{-1} dw) for each fraction.

Alkaline phosphatase (AP) activity

The procedure used to measure AP activity was modified from Donachy et al. (1990). Portions of pyloric caeca, homogenized in ice-cold TMS-HCl buffer (0.02 M Tris; 1.5 mM $MgCl_2$; 10% sucrose), pH 7.5, were centrifuged at 30,000 g (4°C) for 20 min. Enzyme activity of the supernatant was measured as the hydrolysis of *p*-nitrophenylphosphate (*p*-NPhP). Assays were performed at 20°C using a UV-visible spectrophotometer (Varian Cary 1) set at 410 nm. The instrument was operated in the single-beam mode with the display updated every 5 s. Activities were converted from A_{410}/min to nM *p*-NPhP hydrolysed μg^{-1} protein min^{-1} according to assays with pure AP (Sigma). Each assay consisted of 500 μl of buffered substrate solution (0.75M 2-amino-2 methyl-1-propanol; 1 mM *p*-NPhP; 0.25 mM $MgCl_2$) pH 10.3, to which 25 μl of the supernatant from the tissue homogenate were added. Controls consisted of assays run with boiled samples or in the presence of Levamisole, an AP specific inhibitor (Cyboron & Wuthier 1981). Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Data analysis

Significance of the differences between MT concentrations or AP activity was tested by one-way analysis of variance (ANOVA) and by the Tukey's multiple comparison test (Zar 1984).

Results

Metallothioneins

Typical chromatographic elution profiles of the heat-treated cytosol of pyloric caeca of asteroids *Asterias rubens* from an uncontaminated site in SW England (Bigbury) are shown in Figure 2. In the analysed samples, Cu was characteristically associated with the HMM and the LMM pools, while Zn was mainly associated with both the HMM and VLMM pools. The LMM and VLMM pools correspond to expected elution fractions of MT and amino acids/small peptides, respectively. Similar profiles were obtained for field samples and experimentally exposed asteroids.

In contrast, cytosolic distribution of Cd was affected during contamination and varied according to time and level of exposure (Fig 3). No detectable increase was observed for asteroids exposed to 1 μg Cd l^{-1} for 17 days. Cd was accumulated in asteroids exposed to 5 μg Cd l^{-1} for 17 days and was mainly incorporated in the LMM pool of the heat-treated cytosol. Further increase in Cd cytosolic concentrations was observed in pyloric caeca of asteroids exposed to 20 or 200 μg Cd

l^{-1} for 21 days but was not dose-dependent. Indeed, Cd cytosolic concentrations in asteroids exposed to 20 µg Cd l^{-1} were not different than those in asteroids exposed to 200 µg Cd l^{-1}, indicating a saturation of the system (Fig 3 A).

In asteroids exposed to 20 µg Cd l^{-1}, an increase in Cd concentration in the LMM fraction was obvious after 13 days of contamination. The Cd concentration did not increase (and may even have decreased) after 21 days, which is another sign of saturation of the system (Fig 3 B). The major part (>80%) of Cd in the heat-treated extracts of individuals exposed to 20 µg Cd l^{-1} for 21 days was associated with the LMM pool. The principal Cd-binding component had an apparent MM (calculated from Eq. 1) of 22 kDa, whilst a secondary component of 13 kDa was indicated as a shoulder alongside the major peak. Rabbit MT-I chromatographed during our calibration of the column eluted in fraction number 25, indicating an apparent MM of 13.6 kDa. It is possible therefore that the major LMM Cd-binding ligand in *A. rubens* is a dimerised form of MT. To test for polymerization, samples were homogenized in 0.02 M Tris-HCl buffer (pH 8.6) containing 2mM dithiothreitol (DTT). Spectrophotometric patterns and metal distributions in asteroids were little affected as a result of this treatment. However, interference due to the addition of DTT prevented dpp analyses (results not shown).

The effect of heat treatment on the distribution of polarographically-measured thiolic groups, in different cytosolic pools of asteroids exposed to 200 µg Cd l^{-1} for 21 days, is illustrated in Fig 4. Heat treatment was effective in precipitating the bulk of -SH-containing ligands associated with HMM proteins but did not affect the composition of the thiolic-rich LMM component, confirming its thionein-like nature. In heat-treated samples, therefore, the -SH content was predominantly associated with the latter MT pool; it was of similar proportion (66-78%) in all the different field and laboratory-exposed samples analysed. Polarographic responses were not detectable in fractions corresponding to the VLMM pool. Heat treatment therefore leaves a minor, but significant (27-34%), residue of HMM thiolic proteins which interferes with the direct determination of MT in whole-cytosol preparations of pyloric caeca. As they are relatively constant, these interferences could be ignored if comparative estimates of response are all that is required. For more quantitative purposes, however, MT concentrations determined by dpp were corrected for the consistent overestimation attributable to residual HMM thiolic proteins.

MT concentrations in freshly processed and deep-frozen pyloric caeca from control and Cd-exposed asteroids, quantified as described above, against rabbit MT-I as standard, were not significantly different ($p_{ANOVA} \geq 0.2$). The use of deep-frozen material is therefore considered acceptable for this determination. No significant variation ($p_{ANOVA} \geq 0.15$) in MT concentration could be detected in asteroids exposed in laboratory aquaria to 1, 5, 20, 200 µg Cd l^{-1} for up to 21 days, despite the dose-related increases in Cd bioaccumulation shown in Fig 3. However, in asteroids exposed for a longer period in the field, to 20 µg Cd l^{-1}, a significant ($p_{ANOVA} < 0.05$) increase in MT concentration in pyloric caeca was measured after 30 and 45 days (Fig 5). A two-fold increase in MT concentration (maximum = 8.85 ± 3.75 mg MT g^{-1} dw day^{-1}, mean ± SD, n=3) was detected, relative to controls - equivalent to a build up rate of 0.4 mg MT g^{-1} dw. Following transfer of Cd-exposed asteroids to uncontaminated regimens, MT concentrations in pyloric caeca dropped rapidly and by the 8th day of depuration the initial basal level had been reached - indicating a rapid turnover of the metal-binding protein.

The presence of MTs was examined in a natural population of asteroids from the contaminated Sørfjord (SW Norway) to validate the significance of MT induction in *A. rubens* under field conditions. Sørfjord exhibits a clear gradient of heavy metal contamination, concentrations decreasing seawards from the head of the fjord, where 2 smelters have discharged huge quantities of Zn, Cu, Cd, Hg and Pb for decades. The mean concentration of MTs in the pyloric caeca of asteroids varied significantly ($p_{ANOVA}<0.05$) over this gradient (Fig. 6). Highest values, 5.0 - 5.6 mg MT g^{-1} dw, were encountered at stations S2 and S3 (Fig 6; see Fig 1 for locations). S2 is close to one of the smelters, whilst S3 is 12 km further towards the open sea. MT concentrations in pyloric caeca of *A. rubens* sampled close to the first smelter (S1) or outside the fjord (S4) ranged between 2.8 - 3.0 mg MT g^{-1} dw and were similar to levels found at unpolluted sites elsewhere (Fig 6).

Basal levels for MT concentrations in unexposed *A. rubens*, calculated for the different populations used in the present study, were 3.77 ± 1.20 mg MT g^{-1} dw at Bigbury, a fairly pristine bay in SW England, 4.28 ± 1.24 mg MT g^{-1} dw in the Eastern Scheldt (The Netherlands) and 3.00 ± 0.97 mg MT g^{-1} dw in the least impacted outer region of the Sørfjord (SW Norway). Though there would appear to be some variation between populations, reflecting, perhaps, different degrees of background exposure, these means are not statistically different from each other ($p_{ANOVA}=0.06$).

Alkaline phosphatase activity

The specific activity of alkaline phosphatase (AP) in pyloric caeca of *A. rubens* was measured in all samples processed for MT studies. The enzyme did not appear to be affected in asteroids experimentally exposed to Cd ($p_{ANOVA}\geq0.2$). The mean activity in these samples was 13.78 ± 5.37 nM *p*-NPhP hydrolysed μg^{-1} proteins min^{-1}. As observed for MT, storage of material at -80°C had little effect on AP behaviour: in asteroids collected at the contaminated field location (Sørfjord), AP activity at stations S3 and S4 was not significantly different from freshly dissected control asteroids (Fig 6). However, a significant decrease of AP activity of 45%, relative to controls, was measured in asteroids collected in stations S1 and S2 (mean level: 7.55 ± 3.33 nM *p*-NPhP hydrolysed μg^{-1} proteins min^{-1}).

Discussion

The present study quantifies for the first time, to our knowledge, concentrations of inducible Cd-binding proteins of a metallothionein (MT) -like nature in an echinoderm species, together with their expression during experimental exposure to Cd and to a metal pollution gradient in the field. Full characterisation of the protein has still to be carried out; furthermore the optical features characteristic of the metal thiolate complex (high 254/280 nm absorbance, Viarengo & Nott 1993) were not observed, even in exposed asteroids. Nevertheless, the heat-stable, thiolic-rich, Cd-binding proteins present in pyloric caeca of *Asterias rubens* possess a number of chemical properties which are consistent with those of MT, as described by Hamer (1986). Since it is generally accepted that the definition of MTs includes all proteins which are similar to mammalian MTs, even if one or more of the characteristics are absent (Stone & Overnell 1985), this term will be attributed to the LMM Cd-binding protein described here.

According to results based on (1) cytosolic distribution of Cd (2) heat treatment and (3) thiol content, MT in pyloric caeca of *A. rubens* has apparent MM variants of approximately 13 and 22 kDa. This would indicate that MTs occur as monomers and dimers in asteroids, or that partial polymerization takes place during sample processing. The likelihood of the latter event is not considered to be significant as the apparent molecular mass of the rabbit MT-I standard, determined using the same procedure, was close to the expected value of 10 Kda. Nevertheless, the possible production of artefacts during sample preparation (arising from the formation of disulphidic links between the thiolic groups of MT molecules, and their subsequent polymerization to higher molecular mass forms) was tested. Samples were homogenized in 0.02 M Tris-HCl buffer (pH 8.6) containing 2mM dithiothreitol (DTT), an antioxidant that prevents polymerization (see, for example, Pavicic et al. 1993). Spectrophotometric patterns and metal distributions in asteroids were little affected as a result of this treatment showing that dimerization was uncommon during sample processing, however, confirming that dimerization may occur *in vivo*. Unfortunately, the addition of thiol compounds in the buffer perturbs polarographic analysis and, therefore, the inclusion of DTT seems inadvisable when used in combination with this electrochemical technique. The occurrence of two molecular mass classes of MT seems to be widespread in marine organisms though genuine dimerization of subunits has been questioned (Frazier et al. 1985). Determinations of actual molecular mass of MT, (based on amino acid composition) and apparent size estimations determined by gel filtration, generally differ (Carpene et al. 1983, Frazier et al. 1985). The prolate ellipsoid form of MTs (Hamer 1986) decreases retention time on size-exclusion chromatography, compared with a globular protein that would have the same primary structure, thereby giving rise to an overestimation of molecular mass (George & Langston 1994). Based on nucleotidic sequence of echinoid MT mRNA (Nemer et al. 1985), on the low diversity that may be presumed among echinoderm species and without taking into account possible post-translational modifications of MTs (e.g. glycosylation), the actual molecular mass of *A. rubens* MT (monomer) may be close to 8.5 kDa.

MTs occurence in echinoderms has been established from the sequencing of MT genes from echinoid spermatozoa (Nemer et al. 1985). Chromatographic evidence has confirmed the presence of MT-like proteins in both echinoids and asteroids (Jenkins et al. 1982, Besten et al. 1990). However, the degree of MT induction in echinoderms may be species and tissue-dependent. Thus, whilst MT occurs in the ovaries and sperm from echinoids (Nemer et al. 1991; Jenkins et al. 1982; Ohtake et al. 1983), Besten et al. (1990) reported the absence of similar proteins in the gonads of *A. rubens*, describing the pyloric caeca as the main producer of MT-like proteins in this species.

It is important to assess the current results in relation to more general ecotoxicological concepts. Measurements of MT induction in aquatic organisms have been proposed as a means of early detection of a biological response of a population subjected to pollution from class Ib and IIb metals. Indeed, the early detection of a biological response such as this, and subsequent action to reduce cause, is preferable to, and more economical than, remediation of a damaged ecosystem. Provided that the contaminant-induced variation can be quantified, measurement of MTs could be relevant in this context as a biochemical marker in assessing heavy metal effects (George & Langston 1994, Depledge et al. 1995).

Methods to quantify MTs have been reviewed by Engel & Roesijadi (1987) who listed 4 techniques: (1) size-exclusion chromatography coupled with atomic absorption spectrometry of protein fractions, (2) metal substitution assays, suitable for well characterized MTs, (3) immunoassays, (4) polarographic assays. Measurement of MT mRNA can now be added to this list (George & Olsson 1994). Differential pulse polarography (dpp) is advantageous in being a rapid technique that offers adequate sensitivity and specificity for direct measurement of MT concentrations in marine samples. Its use here, in MT determination in pyloric caeca of *A. rubens* represents the first such attempt in echinoderms, though clearly the technique, as applied to this particular tissue, is not without problems: 27 to 34% of the polarographic response was associated with heat-stable HMM proteins. Therefore a preliminary chromatographic separation of the metal binding components was required for an accurate determination of MT concentration. Chromatographic elution profiles, analysed following laboratory exposure of *A. rubens* to increasing Cd concentrations, indicate that MT in pyloric caeca may become rapidly saturated with Cd. This was observed both during the time-course experiment (20 μg Cd l^{-1}, 0 to 21 days) and the dose - response experiment (1 to 200 μg Cd l^{-1}). As MTs are often described as inducible proteins associated with a metal detoxification response (Hamer 1986, Nemer et al. 1991) our observations on *A. rubens* suggest that induction and net production may be limited, at least in the short term, when compared with a number of other organisms.

A further important feature relevant in considering the use of *A. rubens* MT in water quality monitoring may stem from a slow rate of response to metal challenge coupled with rapid turnover of the protein. An increase in MT concentration in the whole cytosol of *A. rubens* was evident after a 30-day exposure to 20 µg Cd l^{-1}. This long time-lag contrasts with, but may be linked to, the rapid observed degradation of the protein, following removal of metal stress. Kinetics of protein induction and turnover follow closely the patterns observed for total Cd measured in identical sub-samples to those presently analysed (Temara et al. in press). Cd is taken up by pyloric caeca according to a saturation model, equilibrium (maximum concentration 12 µg Cd g^{-1} dw) being reached after 20 days (Bjerregaard 1988, Besten et al. 1990, Temara et al. in press). Excess Cd is partitioned to the endoskeleton where it is stored, presumably in detoxified form. On cessation of Cd exposure, mean total Cd concentration decreases exponentially in pyloric caeca, mirroring the behaviour of MT described above.

It can therefore be hypothesized that, during the initial stages of Cd uptake, some Cd binds to the available MTs that constitute basal levels, presumably by displacement of bio-essential elements. When saturation of these constitutive ligands with Cd is approached - which occurs after 10-20d in asteroids exposed to 20 µg Cd l^{-1} - limited induction of further MT is triggered until saturation at the new, slightly higher level is reached, with endoskeleton acting as a secondary sink for uncomplexed metal (Temara et al. in press). Although no direct evidence could ascertain metal redistribution in pyloric caeca of *A. rubens*, a lack of increase in total Cd concentrations after 20 days of exposure (Temara et al. in press) would indicate a redistribution of Cu and Cd between newly synthesized and constitutive MTs.

This cascade of Cd detoxification mechanisms in *A. rubens* appears to be efficient, as evidenced by the lack of effect of short-term laboratory Cd-exposure on alkaline phosphatase (AP) activity in pyloric caeca. The findings therefore address suggestions proposed by Depledge (1993) and others, that "biomarker"-type reponses (including MT induction) should be linked with some estimate of impairment. In the present study, measurements of the activity of AP - a membrane-bound, polyfunctional Zn^{2+}-containing enzyme (Cyboron & Wuthier 1981) - were used as a means of assessing the functional state of the asteroids. Vallee & Ulmer (1972) reported that Cd will replace all the native Zn atoms of AP in the bacillus *Escherichia coli* (Cd phosphatase being enzymatically inactive). Activity of AP of the fish *Mugil cephalus* is known to be inhibited by Cd *in vitro*, but is protected *in vivo* (Hilmy et al. 1985). A similar situation may account for the relationship between AP activity and MT concentrations found in asteroids during laboratory exposure: potentially toxic free Cd in the cells may be effectively bound by MT, or by other sequestration mechanisms, so preventing any significant change in AP activity.

Rapid catabolism of MT in the pyloric caeca and subsequent loss of Cd during depuration, partly to the body wall, appears to be a specialized feature in asteroids. Cd metabolism in other organisms, for example molluscs, is often typified by very slow elimination of Cd following transfer of animals to clean conditions. Thus in bivalves, although MT degradation does occur (with a half-life of approximately 1 month), free Cd released during this process is responsible for, and becomes sequestered by, newly synthesized MT (Bebianno & Langston 1993, Roesijadi

1994). Bivalves such as *Mytilus edulis* may, consequently, retain body burdens of several hundreds μg Cd g^{-1} dw for considerable periods (half-time 300d) after exposure has ceased (Bebianno & Langston 1992).

The Cd-exposure levels used in our experiments (1 to 200 μg Cd l^{-1} for 17 to 45d) were - especially at the upper end of this range - far in excess of concentrations normally encountered in the field, but were included to represent behaviour under extreme conditions. Even close to outfalls in estuaries, Cd rarely reaches such high concentrations for long periods of time because of tidal mixing. Indeed, there are few examples of field validations using MT concentrations as an indicator of water quality, and those reported usually concern either fish or molluscan species (*for review see* George & Langston 1994). The present data on asteroids collected from a heavy metal contaminated environment are, therefore, unique. MT concentrations in pyloric caeca of *A. rubens* from Sørfjord (SW Norway) were significantly higher at stations S2 and S3 (Fig. 1) and might be expected to reflect metal bioavailability in the environment. However, total metal (Zn, Cd, Cu, Pb) concentration distribution in pyloric caeca of asteroids collected along the fjord (own unpubl data) do not fit MTconcentrations distribution. Possible explanations for this poor correlation include: complex inter-element interactions for uptake sites; responses to metals other than those analysed (including Hg which is dumped at Sørfjord, NSTF 1993); the effect of general stress due to the presence of other pollutants such as organic contaminants (chlorinated biphenyls and polycyclic aromatic hydrocarbons are particularly high in the fjord, NSTF 1993).

Reduction in AP activity in asteroids collected in the field corresponded more directly with sites which were highly contaminated by heavy metals. Indeed, such inhibition was significant in asteroids collected from stations S1 and S2. This implies that the functional state of asteroids is affected in Sørfjord, but on this basis is not directly correlated to MT concentrations. However, in view of the limited number of sites involved, more work is needed to interpret the relationships and significance of these responses.

The current research has established basal levels between 2.5 - 4.5 mg MT g^{-1} dw in pyloric caeca of *A. rubens* collected in relatively non contaminated coastal waters of the NE Atlantic, which are comparable with values for mussels *M. edulis* from similar environments (Bebianno & Langston 1991). It is suggested that MT in unexposed asteroids are involved in metabolism of bio-essential trace elements, particularly Cu, as described for a number of other invertebrate species (*for review see* Roesijadi 1992). MT are involved in Cu and Zn homeostasis in fish and while basal concentrations are somewhat lower than those in bivalves or asteroids, their inducibility is higher. Increases in MT levels range between 7-10 fold during Cd exposure and up to 40-50 fold during Zn exposure (George & Langston 1994). Unfortunately, the mobility of fish limits their potential role in biomonitoring programmes unless they are highly territorial species. The relatively sedentary habit of benthic invertebrates such as bivalves and echinoderms, is advantageous for monitoring purposes in this respect.

Though one may be getting closer towards the use of MT in water quality assessment, a further restriction is imposed by our poor understanding of the causes of physiological variability, as determined by endogenous and environmental parameters (other than metals). MT concentrations in mussels are thought to vary seasonally, by perhaps two-fold, even in a "relatively pristine environment" (Harisson et al. 1988), which is close to the variation reported here for asteroids. Priority should be given to evaluating the seasonal fluctuations in MT concentrations in *A. rubens,* particularly in relation to sexual maturity and life cycle stages. MT genes are known to be induced by steroid hormones (Hamer 1986) and levels of progesterone and oestrogen in pyloric caeca of asteroids follow sex-specific cyclical patterns throughout gametogenesis (Voogt et al. 1985), which could involve MT. Moreover, combined effects of metals and steroids on MT production are not to be neglected, since Voogt et al. (1987) showed that steroid metabolism increases in pyloric caeca of *A. rubens* exposed to Cd. These and other potential sources of variability should be assessed in asteroids for a better understanding of the physiological significance of basal MT levels. This would provide ecotoxicologists with a more rigorously defined base- line against which suspected polluted samples could be assessed.

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High sensitivity of skeletogenesis to Pb in the asteroid *Asterias rubens* (Echinodermata)

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Abstract

The effects of lead bioaccumulation on skeleton morphogenesis was assessed in the common asteroid *Asterias rubens*. Regeneration of adambulacral spines was used as an experimental model. Spine regeneration was initiated in asteroids exposed experimentally to 1, 5, 20 or 50 µg Pb l^{-1} for 15 days. Pb bioaccumulation in body wall, skeleton and adambulacral spines was dose-dependent. Scanning electron microscopy showed that skeleton morphogenesis was affected: trabeculae were shorter and more stocky, secondary bridges were not formed after 15 days, trabeculae outline was irregular and mineral deposition was highly reduced. Size of regenerates was significantly smaller in individuals exposed to concentrations $\geq$ 5 µg Pb l^{-1} (up to 70% reduction). Alkaline phosphatase activity was measured as a marker of the functional state of body wall; it significantly decreased (30% reduction) only in individuals exposed to 50 µg Pb l^{-1}. These results indicate that, in comparison with other phyla, skeletogenesis in asteroids is particularly sensitive to Pb and that Pb toxic effects could arise directly from the incorporation of the metal in the skeleton.

Key words: *Asterias rubens* - Pb exposure - stereom morphogenesis

Introduction

Lead is known to be readily incorporated in calcium carbonate skeletons of many marine organisms (*see e.g.* Sturesson 1976, Shen & Boyle 1988, Beeby & Richmond 1989). This accumulation is principally viewed as a detoxification mechanism (Beeby 1991). This view is supported by the observation that shell growth is not affected in mussels *Mytilus edulis* exposed to 200 µg Pb l^{-1} (Strömgren 1982). However, possible effects of lead on skeletogenesis in other taxa have been poorly investigated.

Among invertebrates, echinoderms are noticeable by the producion of a mesodermal skeleton composed of high-magnesium calcite and made of discrete dermic elements, the ossicles. The mineralized part of the ossicles is shaped in a three-dimensional meshwork of trabeculae, the so-called stereom (*see* Dubois & Chen 1989 for a review). Lead affinity for calcite is high (Morse 1986) and Temara et al (1995) showed *in vitro* that Pb is readily adsorbed on echinoderm skeleton. Echinoderms are known to concentrate Pb in their skeleton (Kröncke 1987, Kersten & Kröncke 1991, Gounin 1992, Lawrence et al 1993) and most of the total Pb body burden in asteroids is present in this compartment (Temara et al 1996). Although the skeleton plays a fundamental structural role in echinoderms, no information is, to our knowledge, available on the effects of such Pb accumulation on skeletogenesis.

Stereom morphogenesis in asteroids has been described during regeneration of adambulacral spines in *Asterias rubens* (Dubois & Jangoux 1990). This experimental model is well representative of the phylum, easily initiated *in vivo* and section or removal of adambulacral spines does not lead to major physiological disorders (Dubois & Jangoux 1990). The goal of the present study is to ascertain qualitative and quantitative effects of Pb on skeletogenesis in echinoderms using regeneration of adambulacral spines of *A. rubens* as a model.

Material & Methods

Individuals of *Asterias rubens* (Linneaus, 1758) were collected in the relatively uncontaminated Eastern Scheldt (Scharendijke, The Netherlands). The asteroids (size = 5 to 7 cm from the tip of the longest arm to the opposite interradius) were kept in aerated closed-circuit marine aquaria (salinity: 33‰, temperature: 12 - 14°C) and fed mussels *ad libitum*. Individuals were tagged with Nil blue sulfate (Kvalvågnæs 1972) to allow individual recognition. The upper half of 125 inner adambulacral spines was severed with fine scissors in 25 asteroids, initiating regeneration.

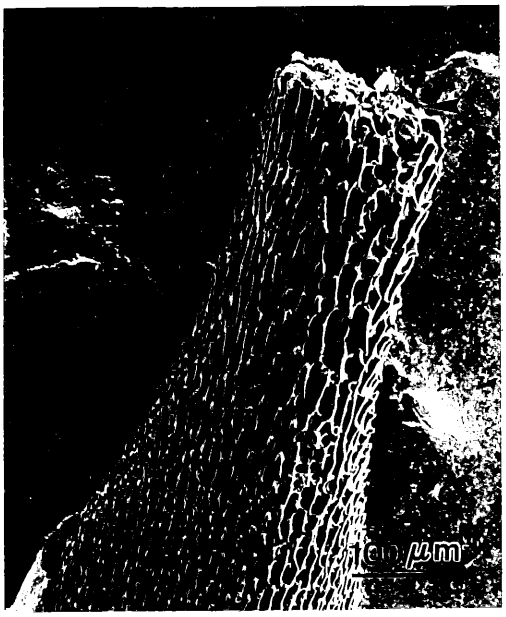
Asteroids were exposed to a range of waterborne Pb concentrations for 15 days from the day the spines were fractured (5 asteroids per exposure type). Nominal contaminating concentrations were 1, 5, 20 and 50 µg Pb l^{-1} [as $Pb(NO_3)_2$, Titrisol, certified quality, Merck]. Water and mussels (both collected in the asteroid sampling site) and Pb were daily renewed. During exposure, 3 asteroids died for unknown reasons in the aquarium containing 1 µg Pb l^{-1}.

Sampling of regenerating spines was performed on asteroids anesthetized with 0.1% propylene phenoxetol - a procedure known not to affect regeneration (Dubois & Jangoux 1990). Spines (n=3) were sampled on each asteroid immediately or 5, 10 and 15 days after fracture. These spines were prepared for scanning electron microscopy (SEM) according to Dubois & Jangoux (1990). They were mounted horizontally on aluminium stubs and size measurements of regenerates were performed on pictures taken with an ISI-DS 130 scanning electron microscope. Calibration of measurements was carried out with electron microscope grids whose mesh size was precisely known (42 µm). Reproductibility of measurements was highly satisfactory as tested on 20 grids (6 measurements on each grid; variation of measurements < 1%; p_{ANOVA} between measurements > 0.99). A grid was mounted on each stub and measurements were calibrated at each used magnification.

At the end of the exposure period (15 days), the asteroids were dissected. One hundred regenerating spines, 100 non-regenerating spines and body wall from each of the 22 asteroids were collected. The segment of body wall tagged with blue sulfate was discarded. The remaining body wall was divided into 3 parts. Skeletal material was isolated from one part by Neutralase grade II (ICN biochemicals) 3‰ in Tris-HCl buffer (0.1 M, pH 7.2) for 48 h at 50°C. Another part was frozen and stored at -80°C for subsequent enzymatic measurements (see below). The third part was dedicated to metal analysis. This third part of body wall, the isolated skeleton and the collected adambulacral spines were prepared for metal analysis as described by Warnau et al (1995). Pb concentrations were measured by spectrometry (flame atomic absorption spectrometer, GBC 906 AA). Detection limit (mean + 3 SD of the blank) equalled 14 µg Pb l^{-1}.

Alkaline phosphatase (AP) activity was measured in portions of the oral body wall according to a procedure modified from Donachy et al (1990). The preparation of samples was carried out on ice. Tissues were manually homogenized in 2.5 volumes TMS-HCl buffer (0.02 M Tris; 1.5 mM $MgCl_2$; 10% sucrose, pH 7.5) and centrifuged at 15,000 g for 20 min. Enzyme activity of the supernatant was measured as the hydrolysis of *p*-nitrophenylphosphate (*p*-NPhP). Assays were performed at 20°C in a PU 8700 - Phillips UV-visible spectrophotometer set at 410 nm. The instrument was operated in the single-beam mode with the display updated every 10 s. Activities were converted from A_{410}/min to nM *p*-NPhP hydrolysed μg^{-1} protein min^{-1} using pure AP (Sigma) for calibration. Each assay consisted of 800 µl of substrate buffer (0.75M 2-amino-2 methyl-1-propanol; 1 mM *p*-NPhP; 0.25 mM $MgCl_2$, pH 10.3), to which 400 µl of the supernatant from the tissue homogenate were added. Controls consisted of assays run with boiled samples or in the presence of levamisole, an AP specific inhibitor (Cyboron & Wuthier 1981). Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Mean regenerate sizes, mean AP activities and mean metal concentrations were compared by one-way ANOVA and the mean multiple comparison Tukey test. Pb concentrations in regenerating and non-regenerating spines were compared by a *t*-test (Zar 1984).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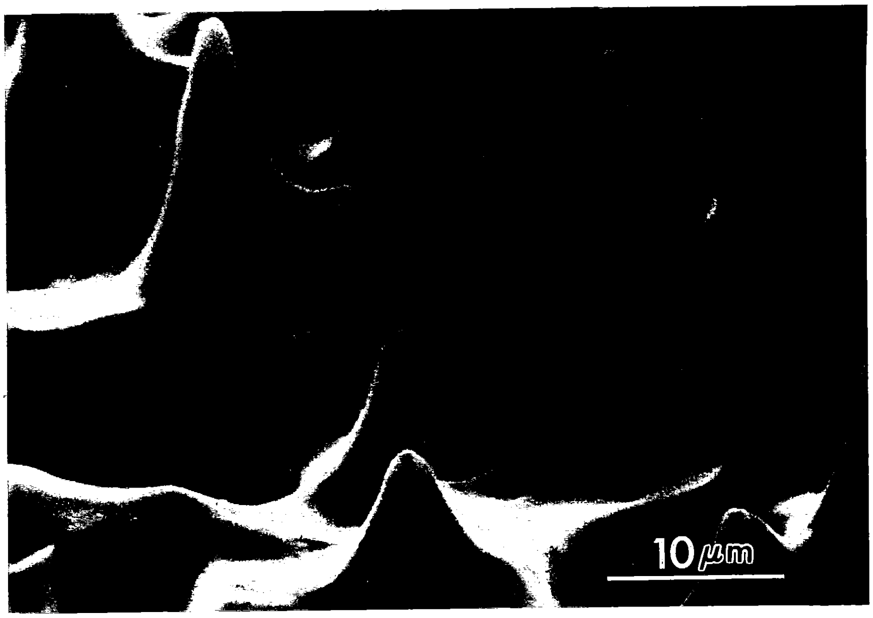
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Results

Lead concentrations in body wall, regenerating and non-regenerating spines and skeleton of asteroids exposed to Pb for 15 days are presented in Table 1. Lead concentrations were low in control asteroids. In body wall and skeleton of exposed asteroids, Pb concentrations increased significantly ($p_{ANOVA} < 0.0001$) with nominal contaminating concentration. Concerning spines, concentrations above the detection limit were only measured in spines of individuals exposed to 20 or 50 μg Pb l^{-1} due to the low amount of material (100 spines weigh approximately 30 μg). Lead concentrations in regenerating spines were not significantly different than those in non-regenerating spines ($p_{t\ test} = 0.9$).

Spines were collected in the course of regeneration and morphology and size of regenerates were studied by scanning electron microscopy. All spines taken from the same individual at the same time presented a similar morphology of the regenerate. Similarly, no difference was found in the morphology of stereom regeneration between individuals exposed to the same level of exposure. The skeleton of a fractured spine is shown in Fig 1. The fracture face of the spine is composed of all the fracture surfaces of the trabeculae. In freshly fractured spines, trabecular fracture surfaces are made of concentric mineral layers (Fig 2). In control asteroids, concentric layers shaded off from the fifth day of regeneration due to the deposition of a fine mineral layer. The latter thickened with time, especially along the margin of the trabeculae, forming a mineral fold (Fig 3). Folds sent out a conical process which developed into a microspine (Fig 4). The latter progressively thickened - becoming a trabecula - and sent out lateral branches. Lateral branches from adjacent trabeculae merged with each other resulting in transverse bridges. First primary bridges were completed by the 10th day in control asteroids (Fig 5). Trabeculae continuously grew in the spine axis and sent out new lateral branches. A general view of a typical 15 days old regenerate in control asteroids is presented in Fig 6. Secondary transverse bridges were formed by the 15th day of regeneration (Fig 7).

In asteroids exposed to the lowest studied concentration (i.e. 1 μg Pb l^{-1}), no effect was observed even after 15 days of exposure and regenerates were similar to control ones. Alterations of stereom morphogenesis did occur in asteroids exposed to concentrations ≥ 5 μg Pb l^{-1}. They were both time and dose-dependent. In asteroids exposed to 5 μg Pb l^{-1} for 15 days, trabeculae were shorter and more stocky than in control spines, trabecula outline was irregular and secondary bridges were not completed. In asteroids exposed to 20 μg Pb l^{-1} for 15 days, more severe damages were observed: trabeculae were shorter and more stocky than in asteroids exposed to 5 μg Pb l^{-1}, trabecula outline was irregular and secondary lateral branches were not present at all (Fig 8-9). In asteroids exposed to 50 μg Pb l^{-1}, microspines from trabecular surfaces were very poorly developed and central concentric layers were still visible after 15 days exposure (Fig 10-11).

Captions.

Figure 6 and 7: Regenerating adambulacral spines in asteroids *Asterias rubens* (controls).

Figure 6: (top left). Regenerating adambulacral spine 15 days after fracture. General view.

Figure 7: (top right). Regenerating adambulacral spine 15 days after fracture. The arrow head points a lateral branche. pb: primary transverse bridge. sb: secondary transverse bridge.

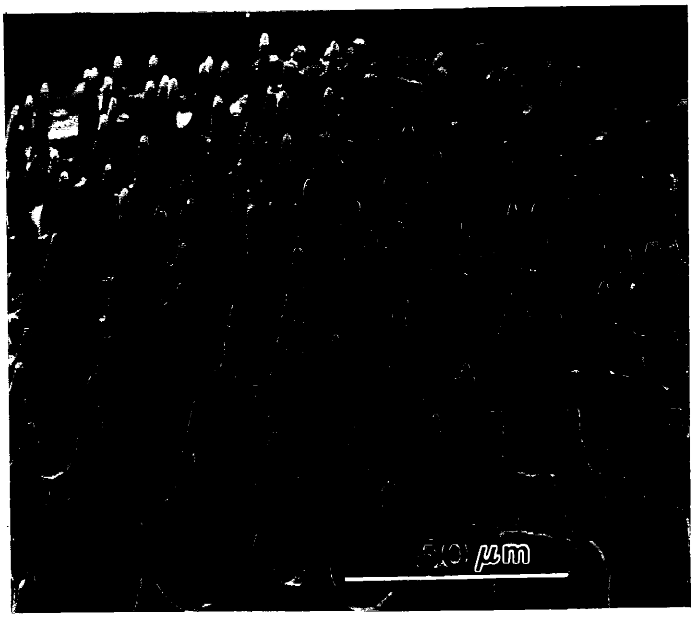
Figure 8 to 11: Regenerating adambulacral spines in asteroids exposed to Pb.

Figure 8: Regenerating adambulacral spine in asteroid exposed to 20 μg Pb l^{-1} for 15 days. General view.

Figure 9: Regenerating adambulacral spine in asteroid exposed to 20 μg Pb l^{-1} for 15 days. The arrow head points a little developed lateral branche.

Figure 10: Regenerating adambulacral spine in asteroid exposed to 50 μg Pb l^{-1} for 15 days. General view.

Figure 11: Regenerating adambulacral spine in asteroid exposed to 50 μg Pb l^{-1} for 15 days. m: microspine. c: concentric mineral layers.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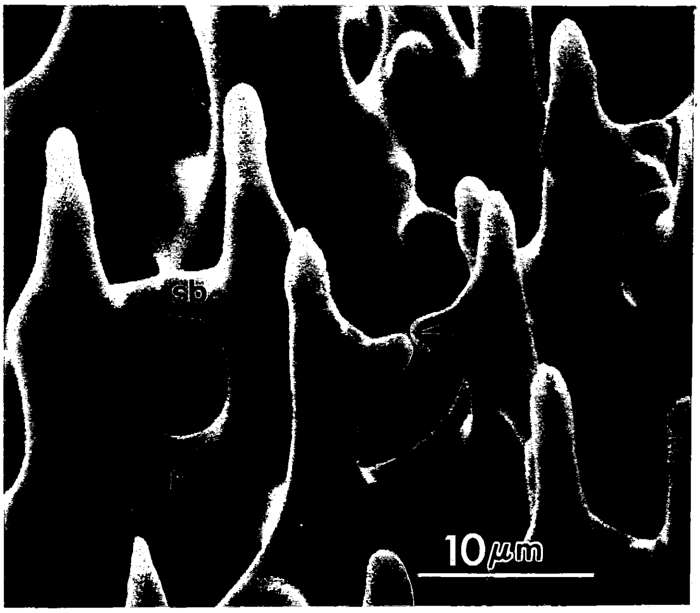
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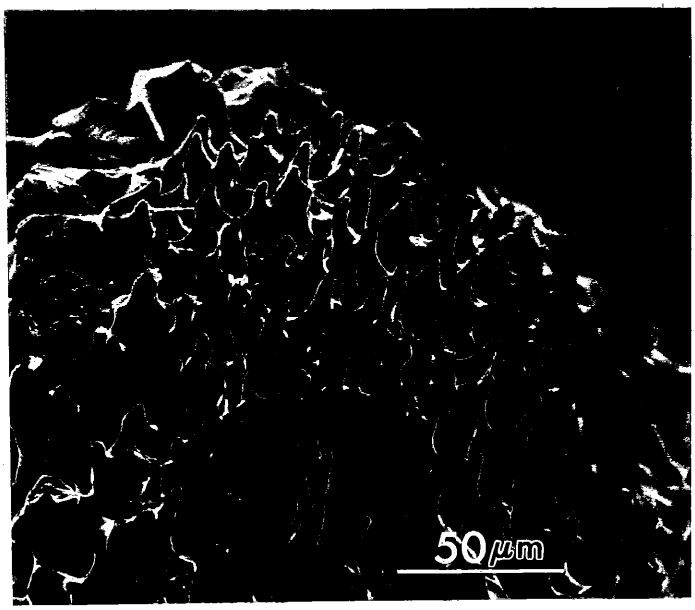
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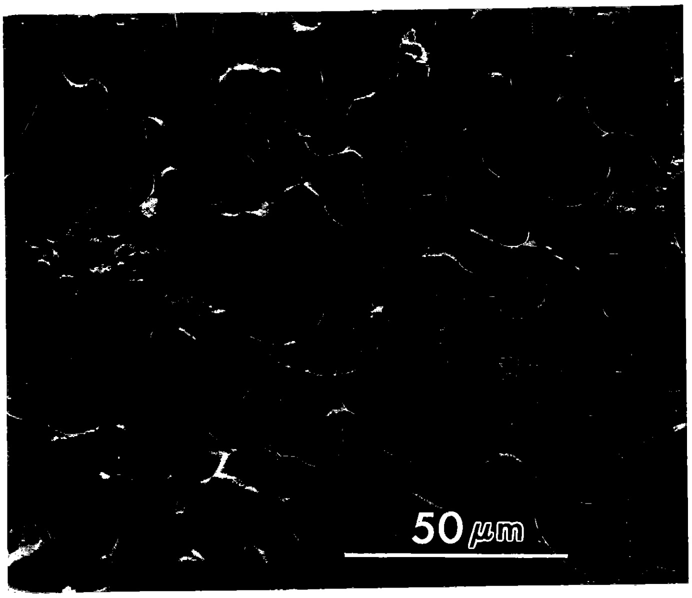
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The size of the regenerates was measured in spines sampled 15 days after fracture (Table 2). Interindividual variations among asteroids exposed to the same Pb concentration were not significant except in asteroids exposed to 50 µg Pb l^{-1} (min and max means: 13.3 - 22.2 µm, $p_{ANOVA} = 0.009$). The mean size of regenerates differed significantly from that of controls in asteroids exposed to concentrations ≥ 5 µg Pb l^{-1}, the effect being dose-dependent. A reduction in regenerate size of 70% was observed in asteroids exposed to 50 µg Pb l^{-1}.

Alkaline phosphatase (AP) activity was measured in oral body wall of asteroids exposed for 15 days to Pb during adambulacral spine regeneration as a marker of the functional state of the body wall. AP activity was significantly affected in asteroids exposed to 50 µg Pb l^{-1} (30% reduction, Table 3).

Discussion

Regeneration of adambulacral spines observed in control asteroids is similar to that described by Dubois & Jangoux (1990). Exposure to 1 µg Pb l^{-1} aparently did not affect the regeneration process. On the contrary, regeneration was affected both qualitatively (structure of trabeculae) and quantitatively (size of regenerates) in asteroids exposed to concentrations ≥ 5 µg Pb l^{-1}. These toxic effects were time and dose-dependent. These results are in accordance with those reported by Warnau & Pagano (1994) who studied Pb embryotoxicity in echinoid larvae. In larvae, the lowest effective concentration (LOEC) measured by these authors was 20 µg Pb l^{-1}, which partially inhibited and disrupted skeletal growth. These toxic effects of Pb appear to be particularly important in echinoderms: in mussels, shell growth is not affected by exposure to 200 µg Pb l^{-1} (Strömgren 1982). Several factors could account for such difference in sensitivity to Pb. Indeed, while mussel shell is an exoskeleton that grows through extracellular deposition of an aragonite/low-magnesium calcite (Lowenstam & Weiner 1989), the endoskeleton of asteroids grows through vesicular deposition of a high-magnesium calcite (Dubois & Chen 1989). This difference in sensitivity between benthic organisms that can live in same ecosystems illustrates the diversity of pollutant effects in marine communities and stresses the importance of developing multi-species toxicity tests. Since relatively high Pb LOEC are reported here (5 µg Pb l^{-1}) and by Warnau & Pagano (1994) (20 µg Pb l^{-1}), direct short-term effects of Pb on echinoderm skeletal biomineralization in the field are likely to be limited to hot spots were high concentrations of dissolved contaminants occur. In asteroids, toxic effects of Pb could arise directly from its incorporation into the skeleton or could alter earlier metabolic pathways and exert a rather indirect deleterious effect. Interestingly, alkaline phosphatase activity did not decrease in asteroids exposed to 5 µg Pb l^{-1} or 20 µg Pb l^{-1} while these asteroids experienced significant alterations of their skeletogenesis. In asteroids, alkaline phosphatase is suggested to be involved in cell division and differentiation associated to wound healing and initiation of regeneration processes (Donachy et al 1990). The present observation thus suggests that Pb does not affect the overall functionning of the body wall at the concentration of 5 µg Pb l^{-1} and 20 µg Pb l^{-1}. On the contrary, it pleads in favour of a direct effect of Pb on skeletogenesis as measurements showed that Pb effectively arrived to the skeleton.

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Temporal and spatial trends of heavy metal contamination in Sørfjord (SW Norway) bio-indicated by the common asteroid *Asterias rubens* (Echinodermata)

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Manuscript submitted to *Mar Ecol Prog Ser*

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Abstract

Asteroids *Asterias rubens* were collected in August 1995 in Sørfjord (SW Norway), a fjord into which heavy metals have been discharged for more than 65 years from smelters located at its head. Discharges have regularly decreased since 1986 and, in 1992, further action was taken to reduce bioavailability of metals by capping the hot spot in the vicinity of the major smelter. Asteroids of the largest size-class were collected at four sites spaced out from the head to the opening of the fjord. In addition, asteroids of different sizes (ages) were collected at the head of the fjord. Concentrations of Zn, Pb and Cd were measured in the pyloric caeca, the body wall and the skeleton of these asteroids. Spatial gradients of heavy metal contamination along the fjord was bioindicated by the asteroid body compartments as metal concentrations were consistently higher (1.7 to 8.3 times) in large-size asteroids sampled closer to the smelters. Difference in the indications given by the pyloric caeca (known as a short-term bioindicator) and the body wall and the skeleton (known as long-term bioindicators) show that bioavailability of metals has decreased over the last years. This is confirmed by the observation that lead concentrations in the skeleton of large asteroids (12 - 14 cm; 154 ± 63 μg Pb g^{-1} dw) were 8 times higher than those in the skeleton of juvenile individuals (0.5 - 4 cm; 19 ± 8 μg Pb g^{-1} dw) in the population located at the head of the fjord. Asteroids of 12 - 14 cm were likely to be 4 - 7 years old (*i.e.* born before the last remedial action) while small asteroids have grown later on (*i.e.* after the remedial action). Smaller size and unusual reduction of the skeleton of adult individuals at the head of the fjord were observed and are proposed to be due to heavy metal pollution. High metal concentrations in asteroids collected at the head of the fjord suggest that bioavailability was still rather high at the time of sampling.

Key words: Heavy metals - Bioindicator - *Asterias rubens* - Sørfjord - Skeleton

Introduction

Heavy metal contaminants introduced into marine systems are mostly concentrated in coastal areas in the vicinity of densely populated and industrialized regions (Fowler 1990). A substantial part of these contaminants are associated with sediments which thus constitute an important secundary source of contamination for littoral ecosystems, even after the primary source had vanished. According to the North Sea Task Force Quality Status Report - NSTF - (1993), the possible impact of contaminated sediments and the speed of recovery of such contaminated areas "remain to be clarified". Sørfjord (SW Norway) is a well suited site to tackle this problem. Sørfjord is an exceptional hot spot where levels of Cd, Hg and Pb in mussels and fish resulted in public advice to regulate human consumption (NSTF 1993). This fjord of 37 km long, 1-2 km wide and 390 m deep holds at its head 3 large smelters, including a zinc plant that has discharged huge quantities of Zn, Cd, Hg and Pb 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 (Naes & Rygg 1982). Inputs were significantly reduced in 1986 due to a drastic reduction in the discharge of metal-loaded residues into the fjord (NSTF 1993, Skei 1993). Figures for 1994 indicated annual discharges of 63.3 tons of Zn, 4.4 tons of Pb and 0.95 tons of Cd (Skei 1995). Metal pollutants are trapped in sediments of the fjord, limiting net efflux to the open sea but allowing any internal recycling (Skei 1981). Release of these sediment-associated metals is significant and is stimulated by the activity of the benthos (Skei 1992). As a consequence, heavy metal concentrations in Sørfjord surface water and biota were not affected to any large extent by the 1986 reduction of discharges. As a remedial to this, the hot spot close to the main smelter was capped in 1992 by a membrane that covered the most contaminated shallow sediment (< 10 m water depth) (Skei 1993).

The present situation in the fjord is that heavy metals in sediments show a clear gradient from high concentrations in inner fjord to basal levels at the outermost end opening on the sea (NSTF 1993). Analyses of different biological samples collected along the fjord give contrasted results according to the considered species. Heavy metal concentrations in cods (*Gadus morhua*) caught at the head of the fjord were not significantly different from those in individuals collected at the opening of the fjord. In mussels (*Mytilus edulis*), concentrations departed from basal levels (ratio: 26x for Cd, 4x for Pb, 2x for Zn) and were identically high all along the fjord (NSTF 1993). Such organisms thus failed to reveal the contamination gradient along the fjord. The efficiency of the remedial actions thus appears to be unclear. This appeals further work using other indicators of metal contamination. Outside fishes and mussels, the commonest macrobenthic metazoan present all along the fjord is the asteroid *Astèrias rubens*.

The present work will use *A. rubens* as a bioindicator of metal contamination in Sørfjord. This benthic predator proved as an efficient biomonitoring tool in studies on heavy metal contamination, the pyloric caeca providing information at short-term (days-weeks) and the skeleton at long-term (months-years) (Bjerregaard 1988, Temara et al. 1993, Temara et al. in press). Recent data concerning *A. rubens* populations in Sørfjord are missing, but these were reported earlier to be highly contaminated by heavy metals (Bryan 1984). The aim is to assess metal contamination in the fjord at the geographical (distance from the head of the fjord) and temporal (before and after the placement of the membrane) scales in order to evaluate the bioavailability of metal contaminants to the macrobenthos.

Material and Methods

Sampling and sample preparation. Asteroids *Asterias rubens* (Linneaus, 1758) were collected by SCUBA divers in August 1995 along Sørfjord (SW Norway). Sampling stations (S1, S2, S3, S4) are located in Figure 1. S1 is situated 100 m from the zinc smelter and is refered to as the hot spot (Skei 1992). S2 is located in front of a titanium smelter. S3 is 12 km further North and S4 is in the neighbouring fjord towards the open sea. Ten asteroids belonging to the largest size class of the considered population (16 - 22 cm from the tip of the longest arm to the opposite interradius) were collected at stations S2, S3, S4. Eighty asteroids representing the whole size range of the population (0.5 - 14 cm) were collected at S1. Asteroids were collected below the density boundary (at -5 to - 10 m) together with water from this depth. They were measured and dissected immediately after collection in the same water. At the sampling period, asteroids had already spawned and the gonads were reduced to the genital cords. Two body compartments were thus separated: the pyloric caeca and the body wall. Samples were frozen (-20°C), then dried (48h, 85°C) and weighed. The skeleton was then separated from of a part of the body wall by proteinase cleaning (Dubois & Jangoux 1985). The procedure was shown not to affect the heavy metal content of the skeleton (Temara et al. 1993). The mass ratio between the skeleton and the body wall was calculated for the largest size class in each station (% of the body wall dry weight).

Metal and data analyses. Concentrations of Cd, Zn and Pb in the body compartments of the asteroids were measured by atomic absorption spectrometry (GBC 906 AA spectrophotometer) after acid digestion of the samples (Temara et al. in press). The reproductibility of the methodology was determined out using an internal standard (homogenized powder of the body wall of asteroids collected at a clean site in Eastern Scheldt, The Netherlands). Three samples of the internal standard were analysed with each sample set. The variations of these measurements (n=24) were low (always within mean ± 2 SD of the first set values). Detection limits (mean + 3 SD of the blank) equalled: 2 μg Zn l^{-1}, 12 μg Cd l^{-1}, 10 μg Pb l^{-1}. Mean concentrations in every compartment were compared by 1-way ANOVA and a mean multiple comparison test (Tukey). Proportions of the skeleton in the body wall were also compared by 1-way ANOVA and a mean

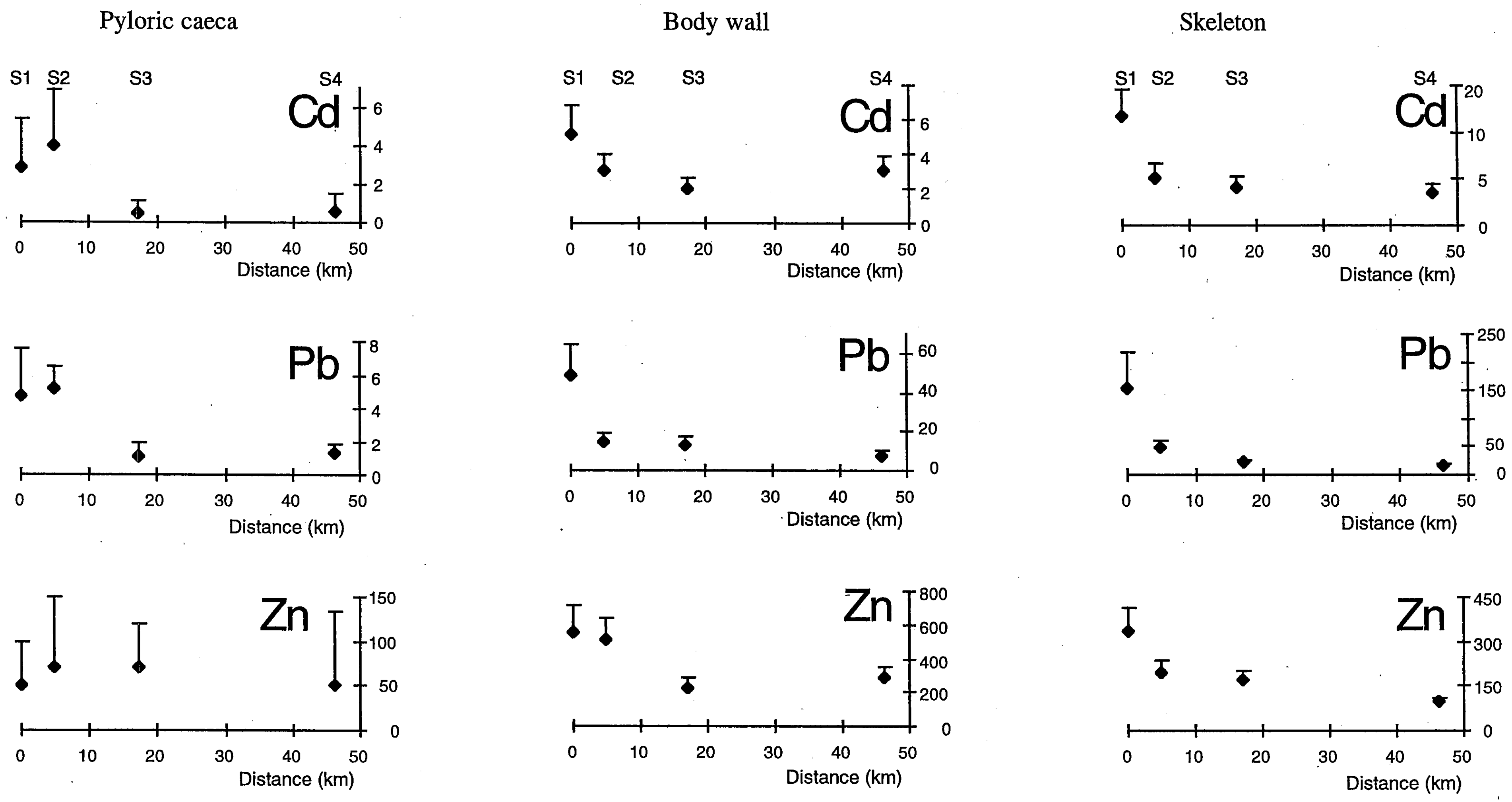


Figure 2. *Asterias rubens*. Heavy metal concentrations (µg metal g^{-1} dw; mean + SD, n=10) in body compartments of adult asteroids as a function of the distance from the head of the fjord (km). S1: 0.1 km, S2: 5 km, S3: 17 km, S4: 47 km.

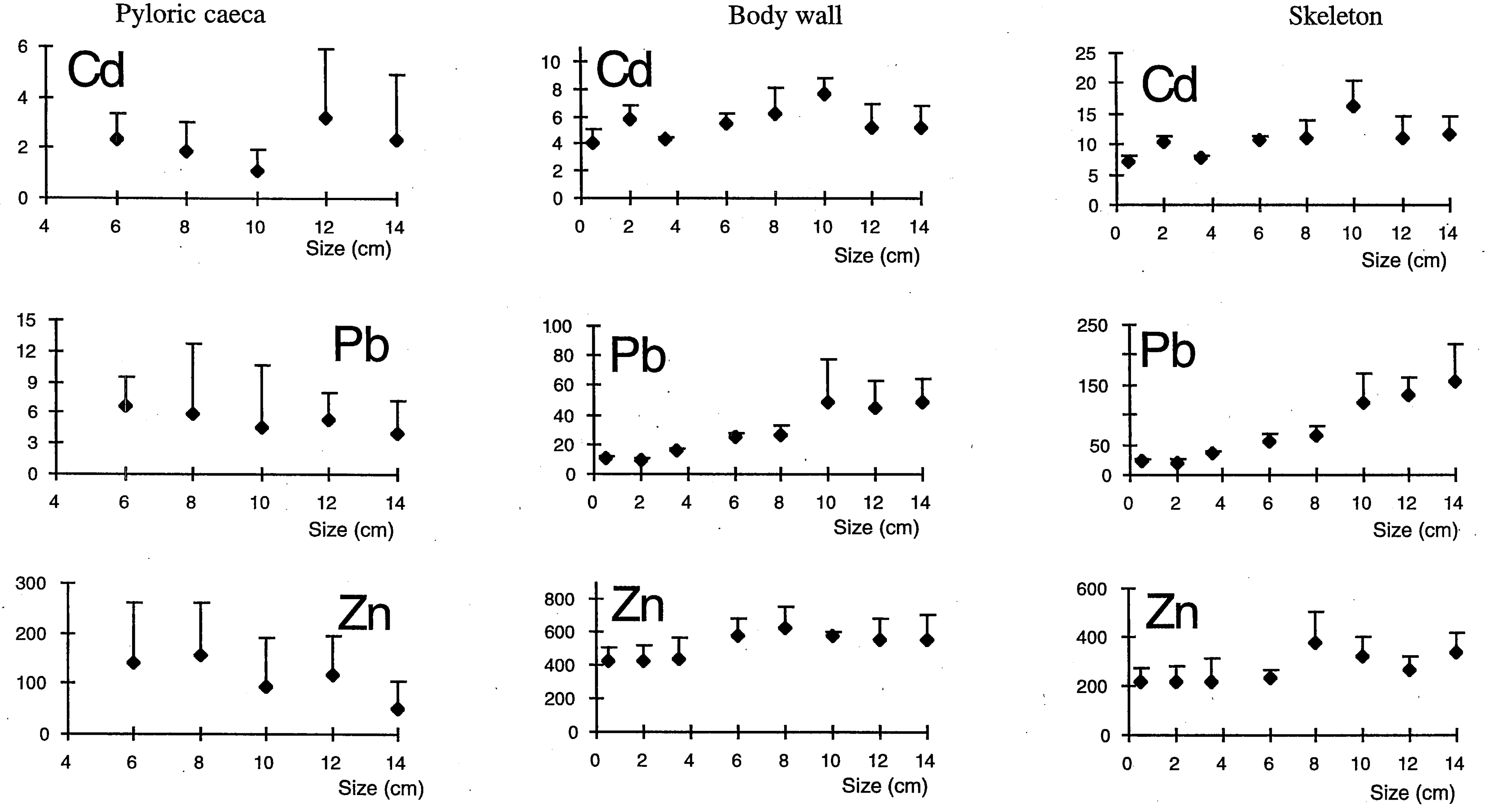
multiple comparison test (Tukey) after arcsine transformation of the data (Zar 1984). The level of significance was set at $\alpha = 0.05$.

Results

Heavy metal concentrations in the body compartments of *Asterias rubens* (large adult specimens) collected at the different sampling stations along Sørfjord are presented in Figure 2; results of statistical comparisons are in Table 1. (Concentrations mentioned hereafter are means).
In the pyloric caeca, concentrations of Pb and Cd were significantly different between asteroids from different stations ($p_{ANOVA} \leq 0.006$). There was a decreasing trend from 3 - 6 µg metal g^{-1} dw in populations close to the smelters (S1 and S2) to 0.5 - 1 µg metal g^{-1} dw in populations located further away (S3 and S4). No significant difference was observed for mean Zn concentrations along the fjord (50 - 70 µg Zn g^{-1} dw). However, there was a high variability between individuals. In the body wall, Cd and Pb concentrations were significantly higher in asteroids collected at S1 (5 and 49 µg metal g^{-1} dw, respectively). Zn concentrations in asteroids collected at S1 and S2 (512 - 555 µg Zn g^{-1} dw) were significantly higher than at S3 and S4 (226 - 276 µg Zn g^{-1} dw). In the skeleton, the general decrease from inner to outer fjord was steeper. Concentrations of Cd, Pb and Zn were significantly higher in asteroids collected at S1 (12, 63, 333 µg metal g^{-1} dw, respectively). Intermediate concentrations were measured in asteroids collected at S2 (5, 47, 194 µg metal g^{-1} dw, respectively). Asteroids collected at S3 had either intermediate concentrations (4 µg Cd g^{-1} dw, 173 µg Zn g^{-1} dw) or lower concentrations (22 µg Pb g^{-1} dw). Lower concentrations were measured in asteroids collected at S4 (3, 16, 96 µg metal g^{-1} dw, respectively) (high, intermediate and lower concentrations are defined according to Tukey test).

In the most contaminated station (S1), metal concentrations were measured in asteroids belonging to the whole size range of the population (0.5 - 14 cm) (Fig 3). In the pyloric caeca, concentrations of Cd, Pb and Zn did not vary significantly with size ($p_{ANOVA} \geq 0.2$). In the body wall and the skeleton, Cd and Zn concentrations did not vary significantly with size ($p_{ANOVA} \geq 0.2$). On the contrary, mean Pb concentration in the two latter compartments varied significantly with size ($p \leq 0.003$) and showed a clear increasing trend. Pb concentrations in the skeleton of the largest individuals (12 - 14 cm; 132-154 µg Pb g^{-1} dw) were up to 8-fold higher than those in the smallest individuals (0.5 - 4 cm; 15-22 µg Pb g^{-1} dw). The steepest difference in concentration occured in individuals of 8 - 10 cm and 10-12 cm.

The size of the largest individuals in S1 was smaller than that of individuals in other stations (12 - 14 cm *versus* 16 - 22 cm). Asteroids belonging to the largest size class in S4 were abundant and measured 18 - 22 cm. Sampling of large asteroids in S1 required an effort 3 - 4 times longer than


Pyloric caeca

Body wall

Skeleton

Figure 3. *Asterias rubens*. Allometric variations of heavy metal concentrations (µg metal g^{-1} dw; mean + SD, n = 10) in body compartments of asteroids collected in S1.

in any other station: they were rare and size never exceeded 14 cm. Proportion of the skeleton within the body wall was calculated for each sampling station. Asteroids from the most contaminated stations (S1, S2) produced less skeleton (mean back transformed data: 34 and 35 %, respectively) than asteroids collected further towards the open sea (S3 and S4; 48 and 51 %, respectively) ($p_{ANOVA}= 0.01$).

Discussion

The asteroids collected in various sites along Sørfjord showed different levels of metal contamination. The amplitude and direction of these variations differed according to the considered metal and the body compartment. Cd and Pb concentrations in the pyloric caeca allow to separate asteroids from stations S1 and S2 on one hand and asteroids from stations S3 and S4 on the other hand. Within both groups, concentrations were not significantly different. Concentrations of Cd, Pb and Zn in the body wall and the skeleton ranked asteroids from the different stations in a gradient decreasing from S1 to S4, S1 harbouring much more contaminated animals.

Uptake of Pb and Cd by the body compartments of *A. rubens* were shown to be directly related to the concentration of these metals in sea water (Bjerregaard 1988, Temara et al. in press, unpubl. data). More precisely, the pyloric caeca were shown to react quickly to environmental contamination (rapid uptake and loss) while the body wall and the associated endoskeleton rather quickly accumulate metals but show long retention times (Temara et al. in press, unpubl. data). The pyloric caeca were thus proposed as a short-term (days-weeks) bioindicator of metal contamination while the skeleton was regarded as a long-term, time-integrating bioindicator. One can thus assume that Cd and Pb concentrations in the pyloric caeca of *A. rubens* reflect the current status of contamination in the fjord while concentrations in the skeleton integrate metal contamination in the fjord over the life-time of the animal. Consistently, metal concentrations in the pyloric caeca did not vary with size (*i.e.* age, see below) in asteroids from S1.

Higher concentrations in the pyloric caeca of asteroids from S1 and S2 thus indicate that a high contamination of the environment still occurs in these stations as compared to S3 and S4. Indeed, low concentrations in asteroids from S4 - *i.e.* out of the fjord - were similar to basal concentrations measured in areas of the North Sea showing background contamination (Everaarts & Fischer 1989, Everaarts et al. 1990, Vyncke et al. 1991, Temara et al. 1993, 1996). Cd concentrations in the pyloric caeca of asteroids collected at S1 and S2 were similar to concentrations at equilibrium in asteroids experimentally exposed to 2-4 μg waterborne Cd l^{-1} (Bjerregaard 1988, Temara et al. in press). As total Cd concentration was 1.9 μg Cd l^{-1} in the low salinity surface water at the vicinity of S1 by the time of asteroid sampling (see Table 2) it is reasonnable to assume that the higher salinity deeper water in which asteroids live showed a

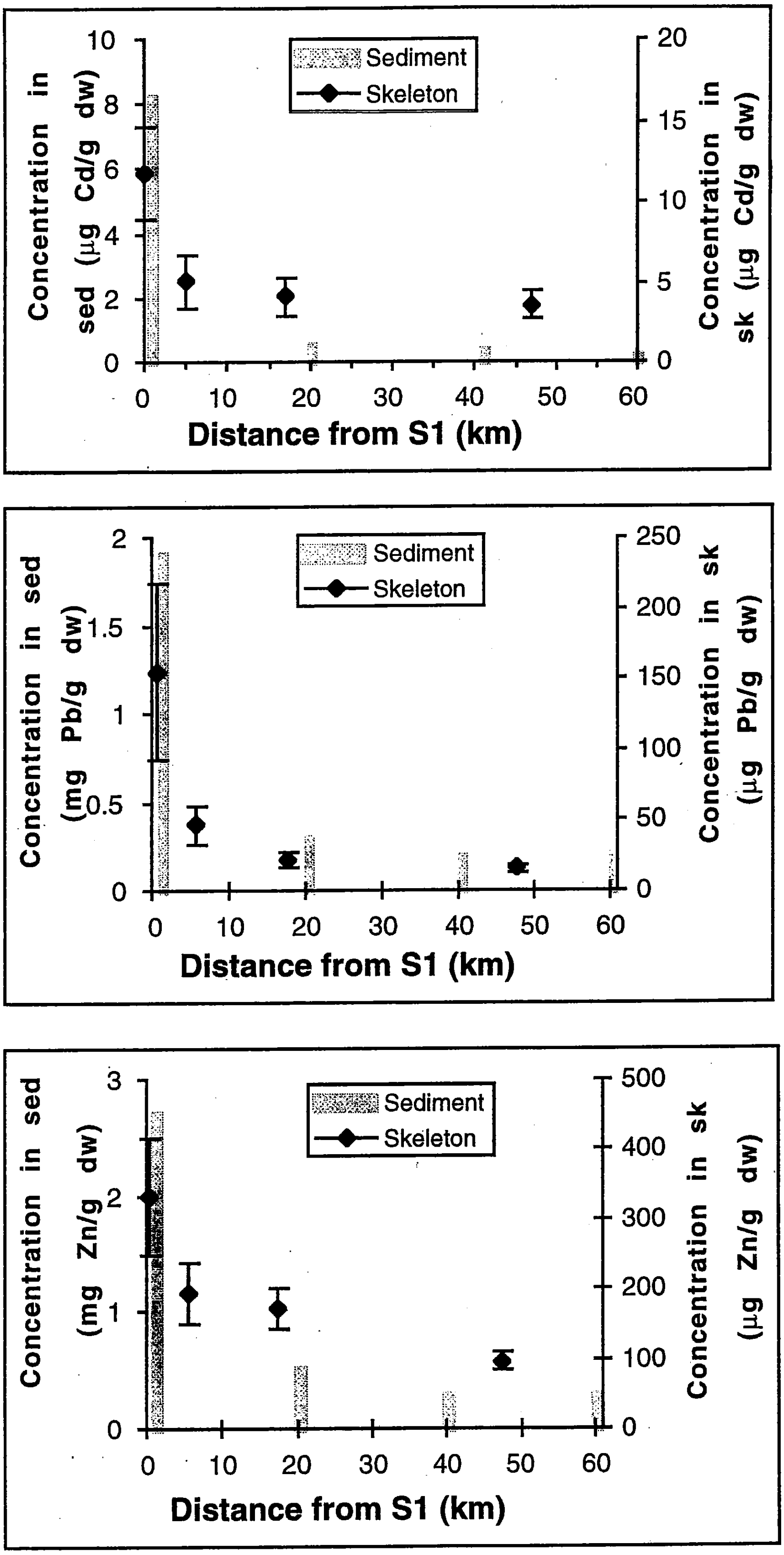


Figure 4. *Asterias rubens*. Heavy metal concentrations (Cd, Pb, Zn) in skeleton (sk) of adult asteroids collected in the present study and in surface sediment (sed) according to NSTF (1993).

lower contamination level. Thus, waterborne Cd does probably not account for the whole contamination of the asteroids. Other possible sources of Cd are food and sediments. Temara et al. (in press) showed that dietary Cd is likely to be the most important source of Cd in contaminated sites. However, in Sørfjord, the prey of *A. rubens* - *Mytilus edulis* - was reported to accumulate high concentrations of Cd all along the fjord, regardless of the distance from the smelters (NSTF 1993). As Cd concentrations in asteroids did decrease with this distance, it can be suggested that sediment-associated metals, which show a clear gradient in the fjord (NSTF 1993), effectively contribute to the body burdens in *A. rubens* in Sørfjord. To our knowledge, direct flows of metals from sediments to *A. rubens* have never been assessed. However, in Sørfjord, asteroids live on fine grain sediments (pers. obs.) and ingest fine particles during extra-oral digestion (Jangoux 1982). This mechanism could result in the contamination of the asteroids by sediment-borne metals.

Contrarily to the indication given by the pyloric caeca, the body wall and the skeleton clearly indicate S1 as the most contaminated station in Sørfjord. Moreover, metal concentrations in the skeleton measured during the present study fit well the available data on geographical variations of heavy metal concentrations in Sørfjord sediments (Fig 4) (data prior to 1993, NSTF 1993). The difference between the largest (*i.e.* the oldest) asteroids of each station reflects a change in the bioavailability of metals to *A. rubens* over the last few years in the fjord (the body wall and the skeleton have integrated metal bioavailability over this period of time). This is confirmed by available data on metal concentrations in low salinity surface water (Table 3) and by the variation of Pb concentrations in the skeleton with size of the asteroids in S1.

A discussion about the relationship between age and size in *A. rubens* can be found elsewhere (Guillou & Guillaumin 1985, Temara et al. 1996). Briefly, asteroids whose size was ≥10 cm were likely to be 4 - 7 years old, while asteroids smaller than 4 cm were born recently (1 - 2 years old). (An accurate estimation of the relationship age - size would require a thorough sampling of the considered population.) Analysis of the allometric relationships in the population of S1 revealed in the body wall and the skeleton highly significant relationships between Pb concentrations and size. As the skeleton accounts for >95% of the total Pb load in the body wall (Temara et al. 1996), one can reasonably assume that variation of Pb concentration in the body wall was a reflection of variation in the skeleton. Three levels of Pb contamination can be distinguished in asteroids of different size. Very high concentrations (50 - 200 µg Pb g^{-1} dw) in asteroids ≥ 10 cm, intermediate concentrations (40 - 70 µg Pb g^{-1} dw) in asteroids of 6 - 8 cm and lower concentrations (15 - 30 µg Pb g^{-1} dw) in asteroids ≤ 3.5 cm. The very high concentrations measured in the skeleton of large asteroids identify environmental contamination as the cause of the observed allometric variation rather than physiological variables. Indeed, allometric variations of Pb concentrations in the skeleton of asteroids from a site showing basal contamination by heavy metals were not significant (Temara et al. 1996). Asteroids ≥ 10 cm in size (4 - 7 years old) would have been exposed to high concentrations for 2 - 4 years before the

Table 1. *Asterias rubens*. Comparisons of heavy metal concentrations in the body compartments of asteroids collected along Sørfjord. Sampling stations are ranked according to increasing metal concentrations in the considered body compartment. p: probability of ANOVA. Concentrations in asteroids sampled in stations with the same superscript are not significantly different (Tukey test) (a<b<c). r: ratio between the highest and the lowest mean concentration. S1, S2, S3, S4: see Fig 1 for localization.

Compartment	**Element**	**Increasing ranking**				**p**	**r**
Pyloric caeca	Cd	$S3^{a}$	$S4^{a}$	$S1^{a,b}$	$S2^{b}$	0.006	7
	Pb	$S3^{a}$	$S4^{a}$	$S1^{b}$	$S2^{b}$	<0.0001	4.4
	Zn					0.3	1
Body wall	Cd	$S3^{a}$	$S2^{b}$	$S4^{b}$	$S1^{c}$	<0.0001	1.7
	Pb	$S4^{a}$	$S3^{a,b}$	$S2^{b}$	$S1^{c}$	<0.0001	4.8
	Zn	$S3^{a}$	$S4^{a}$	$S2^{b}$	$S1^{b}$	<0.0001	2.1
Skeleton	Cd	$S4^{a}$	$S3^{a,b}$	$S2^{b}$	$S1^{c}$	<0.0001	3.4
	Pb	$S4^{a}$	$S3^{a}$	$S2^{b}$	$S1^{c}$	<0.0001	8.3
	Zn	$S4^{a}$	$S3^{b}$	$S2^{b}$	$S1^{c}$	<0.0001	3.4

1993 decrease in contamination level. Asteroids of 6 - 8 cm in size (2 - 4 years old) would have grown during the remedial action. Asteroids ≤ 4 cm in size (1 - 2 years old) would have grown afterwards in a relatively cleaner environment.

The relationship between metal concentration in the skeleton and size was exclusively observed for Pb, an element with a high affinity for carbonate structures (see e.g., Sturesson 1976, Shen and Boyle 1988, Beeby and Richmond 1989, Temara et al. 1995). This indicates that Cd and Zn would have a shorter half-life in the skeleton. Although a low accumulation level of these metals and the subsequent skeletal growth could have resulted in a similar situation, such "dilution" of metals accumulated before 1992 may be ruled out as concentrations in the skeleton of large-size asteroids in S1 are significantly higher than those in asteroids from the other stations.

The effective use of echinoderm skeleton as a bioindicator of temporal variations of environmental contamination is noteworthy. Phillips (1980) reviewed the use of crustacean and mollusc skeletons (shells) as bioindicators and pointed out several conceptual and technical problems in using these taxa. Heavy metal concentrations vary during the moulting cycle of crustaceans and variations are thus likely to be due to different stages of intermolt (see also Devescovi & Lucu C 1995). Similarly, heavy metal content of shells of land gastropods varies with the physiological state as the shell functions as a Ca reservoir and is regularly submitted to resorption processes (Beeby 1991). Shells of barnacles were proposed as a permanent record of environmental changes in trace metal contamination by Watson et al. (1995). However, the high seasonal variability measured by these authors precluded the use of this group as an ideal biomonitoring material. Finally, cleaning the exoskeleton of marine invertebrates from encrusting organisms is not easy. These problems do not exist with the skeleton of echinoderms which is apparently a static structure (Dubois & Chen 1989) and an endoskeleton easily cleaned of surrounded tissues (Dubois & Jangoux 1985).

The elevated concentrations measured in asteroids of Sørfjord tantalizingly stress the question of possible toxic effects of heavy metals in these populations. The smaller size of adult asteroids collected at S1 as well as the poor development of their skeleton could be related - either directly or indirectly - to the extreme conditions that had prevailed before remedial action was taken. Although salinity is usually regarded as the critical environmental parameter in fjords (Skei & Molvaer 1988), Rygg & Skei (1984) pointed out heavy metal load as another determining ecological factor in Norvegian polluted fjords. Salinity does affect growth rate in *A. rubens* (Stickle & Diehl 1987) but data available in litterature indicate that variations of salinity are vertical rather than horizontal in Sørfjord. Indeed, salinity is very low in the top meters during summer when ice and snow of the Folgefonni glacier melts but vertical profiles of salinity are identical all along Sørfjord (Skei et al. 1973). Asteroids are likely to migrate with variation of stratification as they were located just below the density boundary during our sampling. Therefore, salinity is unlikely to account for the differences in asteroid size and skeleton development measured in the present study. Similarly, shortage of food is unlikely, as asteroids

Table 2: Geographical variations of total heavy metal concentrations (n = 5, µg metal l^{-1}) in surface waters along Sørfjord from S1 to the mouth of the fjord in 1995. *: 900 m southwards. (Skei, unpubl. data).

Distance from S1	Pb	Cd	Zn
0	4.6	1.9	61.5
900 m*	0.7	0.8	23.8
7.2 km	0.27	0.28	10.2
22.2 km	0.22	0.23	13.4
39 km	0.15	0.15	9.1

Table 3: Temporal variations of heavy metal concentrations (µg metal l^{-1}) in surface water 900 m southwards S1. n= number of analyses. (Skei, unpubl. data).

Year	n	Pb	Cd	Zn
1989	3	2.87	6.44	943
1990	4	1.41	1	66
1991	4	1.33	1.89	72
1992	6	3.71	0.61	39
1993	6	2.45	0.25	37.5
1994	5	0.48	0.45	42.4
1995	5	0.75	0.8	23.8

in S1 live on a mussel bed (pers. observ.). Rather, heavy metal contamination might have restricted either asteroid recruitment or growth of postmetamorphic asteroids. In the former case, asteroids in S1 would be smaller because younger. In the latter case, asteroids would be smaller in S1 - even if similarly aged - because of delayed growth. It is indeed known that exposure to Cd and Pb affects both larval and adult skeletogenesis in echinoderms at concentrations similar to those found in Sørfjord (Pagano et al. 1982, D'Andrea 1994, Warnau & Pagano 1994, D'Andrea et al. 1996, Temara et al. unpubl.).

It is concluded from the present study that bioavailability of heavy metals to asteroids in S1 did appear to have decreased recently and that capping the hot spot sediment may be recognized as a relatively efficient method to decrease heavy metal bioavailability. However, metal bioavailability in S1 is still high and probably results in toxic effects in *A. rubens*.

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