Bruk av hemocytter fra *Mytilus* sp. i toksisitetstesting og bioovervåkning.

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Forord

Denne masteroppgaven i toksikologi er en del av mastergraden i biologi ved universitet i Oslo. Studiet er delt opp i tre deler og ble utført fra april 2004 til juni 2005. Del 1 består av utvikling av cellekulturer og metoder, del 2 består av *in vitro* toksisitetstesting med utviklede metoder. Del 1 og 2 ble i hovedsak utført ved Norsk Institutt for Vannforskning (NIVA); marin forskningsstasjon Solbergstrand og Oslo-avdelingen. I del ble 3 ble metodene brukt i et feltstudie i Kaštela Bay, Kroatia.

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1. Innledning

Forurensning, og muligheten av skadelige effekter i organismer som følge av miljøgifter i marine miljøer noe man ønsker å unngå. Miljøgifter er kjemikalier som er påvist i miljøet, lite nedbrytbare og kan dermed akkumuleres oppover i næringskjeden (Elliott, 2003). Tilstedeværelse av en miljøgift vil ikke nødvendigvis indikere skade hos individer da dette ikke måler en mulig biologisk respons som konsekvens av eksponering, men konsentrasjon. Tanken om at kjemiske analyser skal kunne forenes med målinger av biologiske effekter har vært foreslått i mer en 25 år (Alabaster & Lloyd, 1980; Sarakinos et al., 2000), men er likevel ikke implementert i overvåkning til tross for at betydelige ressurser er lagt ned. Brukt sammen kan dette gi mer informasjon, samtidig som forståelsen av sammenheng mellom konsentrasjon og effekt økes. Dette studiets hovedmål har vært etablering av uskadelige teknikker for miljøovervåkning og *in vitro* tester av ulike kjemikalier ved å bruke hemocytter fra slekten *Mytilus* som modell. Dette ble gjort ved etablering primære cellekulturer, etablering av ulike toksisitetstester og bruk av metodene i *in vitro* toksisitetstesting og miljøovervåkning.

I 1975 foreslo professor Goldberg (Scripps Institutt for Oseanografi, Universitetet i California) etableringen av et overvåkningsprogram som skulle foreta kjemiske analyser av vev og organer hos skjell og omkringliggende miljø (Goldberg, 2000). Dette skulle være et verktøy til hjelp under overvåkning av marin forurensning hvor man kunne få kvantifisert konsentrasjon, distribusjon og tidsrom for eksponeringer i organismer og marint miljø av en potensiell miljøgift (van der Oost et al., 2003; Widdows & Donkin, 1992). I dag danner dette basis for miljøovervåkning hvor analyser av et utvalg miljøgifters konsentrasjoner i miljø, organismer eller i deres organer blir gjort (Kljakovic-Gaspic et al.; Mackey et al., 1995; Odzak et al., 2000; Wise et al., 1993). Disse dataene blir sammenlignet mot stoffenes grenseverdier, utvalgte referansestajoner eller lignende.

Målinger av biologiske responser mot et kjemikalie på individ, organ, vev eller celle vil kunne gi en mer nyttig informasjon om effekt og eventuelle skadevirkninger ved eksponering (van der Oost et al., 2003). Enhver biologisk respons på dette nivået som demonstrerer en endring fra ”normal” tilstand kan defineres som biomarkør (Walker et al., 2001). Ved bruk av biomarkører vil det være mulig å få tidlige signal

om eventuelle effekter ved forurensinger selv ved utslipp av ukjente stoffer. Dataene vil kunne brukes kvantitativt til prediktering av effekter over eller under det nivået som er studert. Om man i en studie finner ut at en gruppe stoffer svekker immuncellers aktivitet i et individets vil man ut ifra dette kunne anta et svekket immunforsvar som igjen kan antaes å påvirke individets evne til overlevelse.

Til en mulig biomarkør stilles det visse krav. Biomarkørens respons bør være sensitiv for en rekke toksiske stoffer for å kunne gi et tidlig varsel. Data for biomarkøren bør være veldefinert slik at det er mulig å skille mellom naturlig variabilitet og indusert stress som følge av forurensning. I tilegg bør testorganismens biologi og fysiologi være kjent slik at kilder til ukontrollert variasjon kan bli minimert og eksponeringsveier bli kjent. I en toksisitetstest (bioassay) blir et biologisk system som levende celler, organer eller individer eksponert for abiotiske substanser og effekten på testsystemet blir bestemt (NDI, 2005). Testoppsettet (assayet) for kvantifisering av respons bør være pålitelig, relativt billig og enkelt å utføre (van der Oost et al., 2003). Det har også blitt foreslått at en mulig biomarkør ikke bør skade forsøksdyret slik at overvåkning på truede eller beskyttede arter er mulig, og at overvåkningen skal kunne forgå over en lengre periode. En annen fordel ved dette vil være et tidlig varsel om mulige toksiske effekter før en eventuell effekt på individ- eller populasjonsnivå oppstår. Også oppbevaring forenkles da man innenfor akvatisk overvåkning vil slippe store akvariesystem som er kostbare og plasskrevende. Effekter i dyrets naturlige habitat vil kunne måles som kan gi et bedre mål over intensitet av eksponering og effekt av kjemikalier ved enten kunstige blandinger eller ekstrakter slik de fremstår i miljøet (Fossi et al., 1997).

Mange av stoffene som ansees som miljøgifter er relativt nye og det er vanskelig å bestemme hvordan de virker i ulike arter og på celle, organ eller individ. De kan ha alvorlige langtidseffekter eller være svært giftige for miljøet. Selv om en biomarkør viser effekter ved *in vitro* studier og eksponering for stoffer, er det likevel ikke sikkert at man vil få de samme resultater ved bruk av metodene *in vivo* eller i en feltstudie (Reid & MacFarlane, 2003). Ved eksponering *in vivo* eller i sitt naturlige miljø vil organismene ha fordel av et bedre fungerende forsvarsapparat ved en mer kompleks sammensetning av ulike organer og vev enn en eventuell celletype. Stress

som følge av oppbevaring vil også bli unngått. Dette kan skyldes bruk av høyere konsentrasjoner av stoffer enn hva som opptrer i naturen, synergistiske effekter, hvor stoffene reagerer med hverandre og skaper en lavere respons enn forventet, og individuelle forskjeller blant dyrene (Eaton & Klaasen, 2001; Reid & MacFarlane, 2003).

Mytilus sp. som biomarkør kan ha mange fordeler. Artene innenfor slekten *Mytilus* er dominerende medlemmer av kystsamfunn, og har stor geografisk utbredelse. De er også stedbundne, relativt robuste mot en rekke miljøgifter og kan lett transplanteres til områder av interesse. Populasjoner av blåskjell er vanligvis store, og tåler derfor gjentatte prøvetakninger. Blåskjell tar til seg føde ved å filtrere partikler fra sjøvannet og pumper derfor mer eller mindre kontinuerlig store mengder vann. Dette kan føre til en videre oppkonsentrering av stoffer i deres vev og organer. Faktorer som årstid og gyteperiode kan påvirke lipidnivåer i reproduksjons- og fordøyelsesorganer og dermed også relativ distribusjon og akkumulering i vev av lipofile stoffer (Livingstone & Pipe, 1992). Som viktig føde for en rekke dyr vil akkumulering av miljøgifter i blåskjell føre til en videre mulig overføring og oppkonsentrering av miljøgifter oppover i næringskjeden (Widdows & Donkin, 1992).

I bløtdyr består sirkulasjonssystem av hjerte og hemolymfe som inneholder hemocytter. Hemolymfen er et åpent sirkulasjonssystem som bader vevene direkte og består av tre typer hemocytter, granulocytter, små basofiler og makrofagiske celler. Cellenes diameter er mellom 3,9 og 12 µm. Hemocyttene reagerer med fremmede substanser ved fagocytose eller innkapsling. Prosessen virker å være spesifikk og involverer flere steg: Gjenkjennelse av fremmede substanser og celleproliferasjon, bevegelse mot stedet med fremmed substans og til slutt binding og fordøyelse av intracellulære degraderingsprodukter. Ved utveksling av gass med omgivelsene osmotisk regulering, fordeling av næringsstoffer, eliminering av avfall og reparasjon av indre skader spiller også hemocyttene en viktig rolle. Hemocyttene er ikke bundet til hemolymfen, men kan bevege seg fritt inn og ut av sinus og de omkringliggende vev, kappehulrom og fordøyelse. De utgjør sannsynligvis det viktigste indre forsvar i alle bløtdyr (Hill & Welsh, 1966; Martins et al., 2005). Studier utført tidligere har vist at en rekke kjemiske stoffer eller kontaminering av

marine miljø kan ha en ugunstig effekt på muslingers immunsystem (Bihari et al., 2003; Canesi et al., 2005; Canesi et al., 2002; Luengen et al., 2004; Martins et al., 2005; Oliver et al., 2003). Dette kan senke individets overlevelse med en senket resistens mot sykdommer og parasitter (Gosling, 2003).

Til tross for dette er det i mange akvatiske organismer vist evne til overlevelse og reproduksjon i områder med høy grad av kontaminering. Ved kjemiske analyser har det blitt vist at de har et uventet lavere nivå av miljøgifter i vev og organer enn i omkringliggende omgivelser som indikerer et velfungerende forsvarssystem. Det har blitt vist i mange akvatiske organismer at de innehar en mekanisme lignende "multidrug resistans" (MDR) observert i kreftceller som er resistente mot cytostatika (Keppler & Ringwood, 2001; Kurelec, 1992; Kurelec & Pivcevic, 1991; Minier & Galgani, 1995). Induksjon av denne mekanismen, kalt multixenobiotisk resistens i akvatiske organismer (MXR) og multidrug resistens (MDR) i pattedyr, har blitt rapportert i flere studier etter eksponering i laboratorium mot en rekke stoffer, eksponering i miljøet for antropogen kontaminering eller naturlig stress hvor resultatet har vært økt transport av modellsubstrat ut av cellene eller organene (Eufemia & Epel, 2000; Kurelec et al., 1995; Minier et al., 2000; Minier & Moore, 1996; Smital et al., 2003). Ved å inneha en mekanisme som fjerner skadelige substrater fra cellenes indre miljø kan dette forhindre akkumulering av toksiner. MXR rolle som forsvar har vist seg å være skjør da mange ulike klasser med kjemikaler kan virke hemmende på utpumpingen av substrater. Disse refereres til som "chemosensitisers", og kan virke skadelige da de fører til akkumulering av xenobiotiske stoffer (Smital & Kurelec, 1998). Det har vært gjort flere forsøk på å karakterisere disse, og konklusjonen er at de fleste stoffene som kan hemme virker å være hydrofobe og inneholder en karbonylgruppe (Kurelec, 1992). Induksjon eller inhibering av MXR har enda ikke blitt etablert som biomarkør ved overvåkning av marine miljøer (van der Oost et al., 2003).

Dette studiet har hatt som hovedmål å etablere uskadelige teknikker for bioovervåkning og *in vitro* test av ulike kjemikalier ved å bruke hemocytter fra slekten *Mytilus* som modell. Delmål i dette studiet har vært følgende:

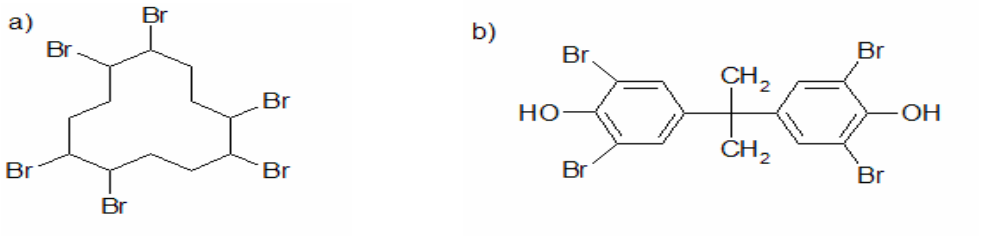
- Etablere primære cellekulturer med hemocytter fra blåskjell og etablere bruk av cellebaserte toksisitetstester (bioassay) med MXR-aktivitet og celleviabilitet som endepunkt.

- Teste hemocytter respons på et utvalg aktuelle miljøgifters effekt på celleviabilitet og induksjon/inhibisjon av MXR for å øke kunnskap om deres effekt på og eventuelle kjønnforskjeller.

- Vurdere bruken og nytten som biomarkør i et feltstudie.

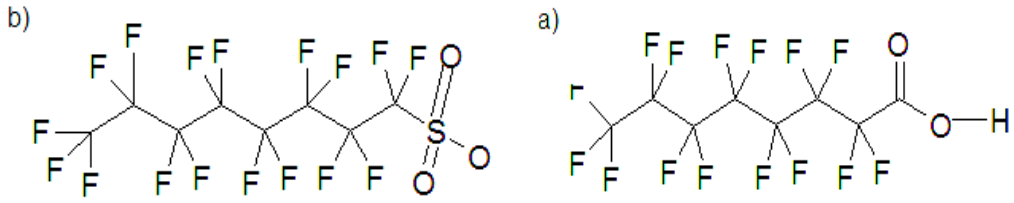
1.2. Forekomst og kjent toksikologi ved testsubstanser

I dette studiet ble fem miljøgifters toksisitet testet *in vitro*. Stoffene hadde til felles en plassering på norske miljøvernmyndigheters liste over prioriterte farlige stoffer, var helse- og miljøfarlige og blir brukt i et slikt omfang at de kan skape problemer på et nasjonalt og internasjonalt nivå. Stoffene på denne listen er persistente og akkumuleres i organismer. Bruk og/eller utslipp av denne gruppen stoffer skal stanses eller reduseres betraktelig innen 2010 (SFT, 2004).



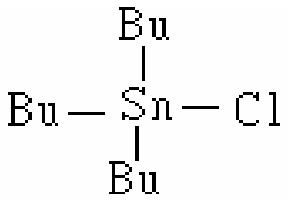
Figur 1 Bromerte flammehemmere. a) Heksabromsyklododekan (HBCD) og b) Tetrabrombisfenol A (TBBPA)

Bromerte flammehemmere er organiske stoffer som tilsettes i en rekke plastkomponenter i produkter som i elektroniske og elektriske produkter (figur 1). Fokus har vært rettet mot denne gruppen stoffer etter oppslag i Aftenposten i 2003 og 2004 om funn av store av denne gruppen i ørret fra Mjøsa (SFT, 2004). Det finnes ca 70 forskjellige stoffer innenfor gruppen. Meget høy akutt giftighet for vannlevende organismer og høyt potensial for bioakkumulering har satt disse stoffene på listen. Det totale forbruket var i 2002 antatt til å være mellom 280-330 tonn i Norge. Tetrabrombisfenol A (TBBPA) er den mest brukte bromerte flammehemmeren, som utgjør ca 2/3 av totalt forbruk. En annen bromert flammehemmer, heksabromsyklododekan (HBCDD) med antatt forbruk i 2002 mellom 20-25 tonn (SFT, 2005). For TBBPA er det funnet opptil 364 ng/g (fett i blåskjell ved Færder fyr, Oslofjorden (Fjeld et al., 2004). Av HBCD har man funnet konsentrasjoner opp til 10 ng/g våtvekt i vev hos blåskjell ved samme sted (Schlabach et al., 2002).



Figur 2. Perflouralkylerte stoffer. a) Heptadekafluorononа syre (PFNA) og b) perfluoroktanylsulfonat (PFOS).

Perflouralkylerte stoffer (PFAS) er samlebetegnelsen på en gruppe kjemiske forbindelser som inneholder perflourstrukturer (se figur 2). De nedbrytes ikke i sure eller basiske miljøer, eller ved høye temperaturer. De er ikke brennbare, og brukes derfor ofte i brannslukningsapparater. Stoffene er vann- og smussavstøtende og brukes derfor også ofte i tekstiler og papir. Foreløpig er ikke disse stoffene forbudt i Norge, men står på miljøvernmyndighetenes liste over prioriterte farlige stoffer da det har vist seg at de akkumuleres i fettvev og virker reproduksjonstoksisk under ammeperioden (SFT, 2004). Utslipp i 2004 var estimert til mellom 12 og 15 tonn. I analyser gjort på blåskjell var konsentrasjonen over deteksjonsgrensa med henholdsvis 78,4 ng /g våtvekt (168,4 nM) blåskjell av PFNA, og 165,3 pg/g våtvekt (0,264 nM) blåskjell av PFOS (Schlabach et al., 2004).



Figur 3. Tributyltinn (TBT); Bu – butylgruppe.

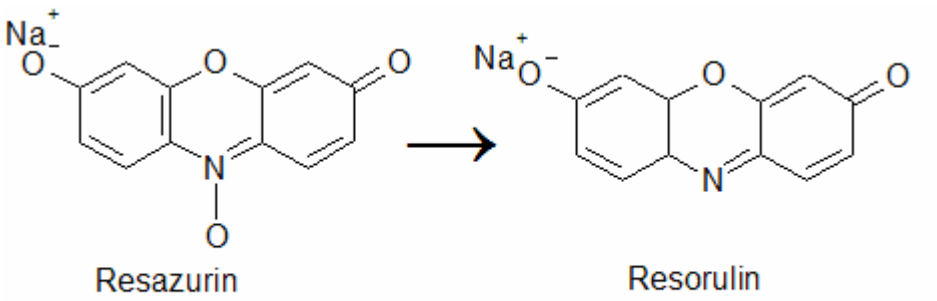
Tributyltinn(TBT)- og trifenyltinnforbindelser (TFT) er kunstig framstilte tinnorganiske forbindelser som i hovedsak har blitt benyttet i bunnstoff i båter. De er nå forbudt (SFT, 2005). Organiske tinnforbindelser er vanligvis ikke-polare og lipofile molekyler (se figur 3). De kan lett interagere med biomembraner og penetrere disse. Det er påvist at de binder seg til biologiske molekyler, forandrer ionebalansen *in vivo* og forårsaker toksisk effekt som skade på mitokondrier ved for eksempel blokkering av ATP syntese (Huang & Wang, 1995). De har også blitt

brukt i produkter som trebeis og tremaling, desinfeksjonsmidler, konserveringsmidler og rengjøringsmidler. Stoffene er tungt nedbrytbare og kan derfor oppkonsentreres i organismer. Forbindelsene opptrer i forhøyede konsentrasjoner i sedimenter nær skipsverft, marinaer og trafikkerte havner og skipsleier. Det er påvist forhøyede konsentrasjoner TBT i blåskjell og purpursnegl i trafikkerte områder langs Norges kyst. Analyser av purpursnegl har vist konsentrasjoner på over 100 ng /gram (0,64 µM) tørrvekt snegl med en klar korrelasjon med grad av imposex. I belastede områder er det observert skader på forplantningsorganer hos purpursnegler med utvikling av penis hos hunner. Årlig overvåking siden 1997 av TBT -nivåene i snegl og blåskjell fra ytre Oslofjord og Haugesundsområdet viser ingen endring til tross for forbud i 1990 mot bruk på båter under 25 m (SFT, 2005). Innen utgangen av 2007 skal all TBT som har vært brukt som bunnstoff fjernes. Utslippene har blitt redusert fra 58 tonn i 1985 til ca. 7 tonn i 2003.

2. Material og metode

I denne oppgaven vil to tester bli brukt for å vurdere hemocytter som markører i toksisitetstesting og i feltstudier. Den ene testen vil vurdere cellenes viabilitet, og den andre evne til å pumpe fremmedstoffer ut av cellene. Bakgrunnen for disse to testene vil bli forklart i de neste to avsnittene.

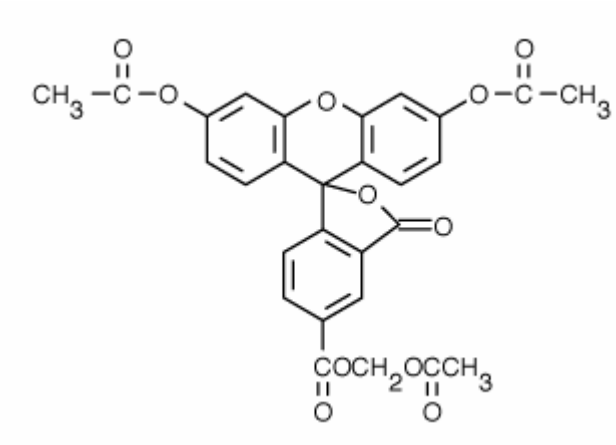
Tester som evaluerer cellers viabilitet har et stort bruksområde innenfor celledyrking og ved bruk av *in vitro* toksikologi. I dette studiet vil de to probene alamar Blue™, og CFDA-AM benyttes som mål på metabolsk aktivitet og membranintegritet bli brukt og utgjøre et mål på cellenes viabilitet. Rezasurin, med kommersielt navn alamar Blue™, reduseres av cellulære reduktaser, sannsynligvis diaforaser eller NADH dehydrogenase i levende celler (se figur 4) til en fluoriserende form, resorulin (O'Brien et al., 2000). En har tidligere trodd at reduksjon av alamar Blue har tatt sted i cellenes mitokondrier, men ingen bevis for dette ble funnet av O'Brien et al (2000). I denne artikkelen vil alamar Blue™ fluorescens vil bli referert til som metabolsk aktivitet. Proben er vannløselig og kan diffundere fritt på tvers av konsentrasjonsgradienter i både redusert og oksidert form.



Figur 4. Reduksjon av alamar Blue i viable celler til et fluorescerende produkt.

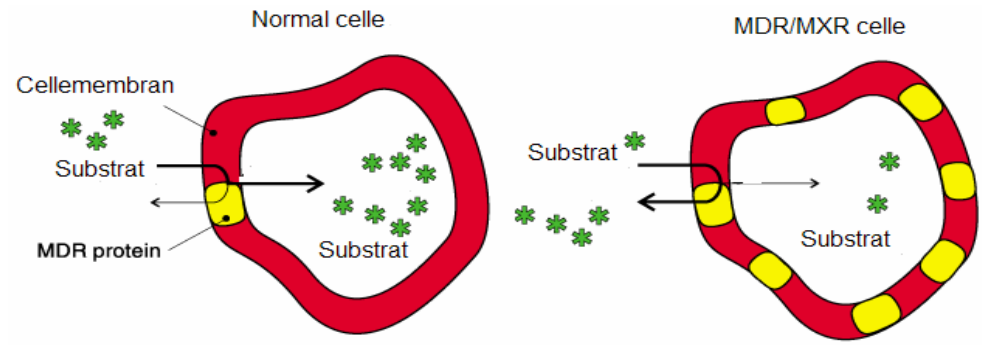
Esterase aktivitet måles av proben 5-karboksyfluorescein-diacetat, acetoksymetyl ester, CFDA-AM. (Se figur 5). Denne proben konverteres av uspesifikke esteraser i levende celler fra et upolart, ufluorescerende fargestoff. Substratet diffunderer raskt inn i cellen, mens produktet diffunderer sakte ut. CFDA-AM leses ved eksitasjon 485 nm og emisjon 530 nm. Målingene vil reflektere membranintegritet av cellene. Dette vil enten skyldes redusert esterase aktivitet ved forstyrrelse av cytoplastisk

miljø ved lavere membranintegritet, eller at metabolisert CFDA-AM kan diffundere raskere fra celler med lavere membranintegritet.



Figur 5. CFDA-AM. Reduseres av esteraser i cytosol.

Fordi de to probene måles ved ulike bølgelengder kan begge fargestoffene brukes samtidig. Dette kan gi et mer helhetlig bilde av kjemikaliers toksiske effekt på celleviabilitet. Probene trenger ikke å fjernes fra brønnene, og testen kan utføres rett etter innkubering uten videre forberedelser (Ganassin et al., 2000).



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Figur 6. Celler med og uten forhøyet MXR aktivitet. Modifisert etter figur i "A Guide to Fluorescent Probes and Labeling Technologies" (Invitrogen, 2005).

I celler med MXR-aktivitet har det blitt vist overeksponering av membranglykoproteinet kalt Pgp (se figur 6), og økte mengder av P170 mRNA. Pgp-proteinet består av 12 transmembrane segmenter som former en substratbindende pore, og to cytoplastiske ATPbindende seter. Denne molekylære strukturen er typisk for medlemmer av ATP-bindende kassett (ABC) protein familie. Over 50 medlemmer av denne familien har blitt karakterisert (Armstrong et al., 2004). Ulike proteiner har blitt påvist å ha ulik substratspesifisitet, og det er

sannsynlig at disse kan virke i samme celle til samme tid og dermed forhindre endogene og eksogene kjemikaliers akkumulering og mulig toksiske effekt (Kurelec, 1992). Cyclosporin A har tidligere blitt vist å hemme proteinet MDR 1 i pattedyr, MK 571 en potent hemmer av MRP1 og verapamil har vist seg å være en potent hemmer av både MRD1 og MRP1(Vellonen et al., 2004). Fjerning av substrater skjer ved en aktiv utpumping via transmembrane pumper (Loscher & Potschka, 2005; Twentyman et al., 1994; Weinstein et al., 1990; Wigler & Patterson, 1993).

2.1. Utvikling av metode (DEL 1)

2.1.1. Forsøksdyr

Det ble benyttet blåskjell av arten *Mytilus edulis* hentet fra NIVA`s marine forskningsstasjon, Solbergstrand, Drøbak i juni. Skjellene ble plukket ved 30 cm dyp ved lavvann, varierte i lengde fra 4-7 cm, og ble oppbevart i en plastikkboks under prøvetaking. Sjøvannet holdt 17˚C og 24 ‰ S.

2.1.2. Prøvetaking av hemocytter

Blåskjellene ble åpnet 1-2 mm med tuppen av en pinsett uten å skade skjellet. Med en 23gauge sprøytespiss og 1 ml kanyle som inneholdt 0,1 ml kald PBS 24 ‰ (85 ml 0, 2 M $Na_2HPO_4 \cdot 12\ H_2O$, 915 ml 0, 2 M $NaH_2PO_4 \cdot 12\ H_2O$, 1000 ml destillert vann og 48 g NaCl) ble hemolymfe fra bakre lukkemuskel hos dyrene prøvetatt og overført til et Nunc 50 ml plastikkrør. Samleprøven ble satt på is, og snudd hvert 2. minutt for å forhindre sammenklistring.

2.1.3. Medium

Cellekonsentrajonen i hemolymfe-PBS løsningen ble bestemt ved hjelp av Multisizer^{TM} Coulter counter. Multisizer ble stilt inn til å registrere partikler innenfor diameter av *M. Edulis* hemocytter (Friebel & Renwrantz, 1995). Etter protokollen ble 200 µl løsning tynnet ut i 9,8 ml isotont destillert, filtrert vann. Tre skyllinger ble gjort for å fjerne uønskede partikler. Når partiklene ble suspendert i den svake elektrolyttløsningen ble de trukket mot og igjennom to elektroder. Forandringen i spenningen mellom de to elektrodene som følge av partiklers gjennomstrømming produserer en pulsering som er proporsjonal med partikkelens tredimensjonale volum. Volum av partiklene som en ønsket å telle ble stilt inn, og hver puls som følge av partikler innenfor denne rekkevidden ble registrert. For kvantifisering av hemocytt-konsentrasjon ble følgende formel brukt:

*Konsentrasjon = n partikler registrert *fortynningsgrad*50*10^3*

To ulike medier ble testet, Leibowitz L-15 (500 ml L-15, 5 ml L-glutamine, 4,5 ml Penicillin Streptomycin Fungicon og 2,5 ml $NaHCO_3$) og PBS. Ved videre

fortynning med enten L-15 eller PBS ble konsentrasjoner på 10 000, 20 000, 40 000 og 80 000 celler/200 µl medium med 16 replikater av hver konsentrasjon. Hemocyttene ble innkubert i mørke ved 15˚C i fem dager slik at cellene skulle få feste seg til celleplatene. Etter dag 5 ble en viabilitetstest utført for å evaluere suksessen av medium og cellekonsentrasjon. Dette ble gjort ved å forsiktig fjerne alt medium med hjelp av en multipipette, og så i mørke tilsette en miksture inneholdende 11,6 µl av proben 4 mM CFDA-AM, 579 µl av proben alamar Blue ™ og 11 ml medium. Cellene ble satt til innkubering i 30 min på en orbital ristemaskin på laveste styrke. Plateleseren CytoFluor ™ 2300 ble satt til eksitasjon og emisjon i bølgelengdepar på 530-590 (alamar Blue) og 485-530 (CFDA-AM). Gjennomsnitt fra blanke brønner ble utregnet og subtrahert fra data

2.1.4. Endring av viabilitet hos hemocytter over tid

Hemocytters evne til overlevelse i en primær cellekultur over lengre perioder ble testet ved å holde cellene i samme medium over 30 dager. Hemolymfe fra 15 dyr ble fortynnet til 200 000 celler/ml PBS og utsådd i 96 brønners celledyrkningsplate. Som blank prøve ble det sådd ut kun PBS i 8 brønner. Dag 1, 5, 10, 15 og 30 ble en viabilitetetstest på 8 minimum replikater av gangen utført (se protokoll i avsnitt 2.1.3.). Etter at probenes fluorescens var avlest ble alt innhold i de testede brønnene fjernet for å unngå mulig ugunstig effekt på gjenværende celler.

2.1.5. Viabilitetetstest av hemocytter, standardisering

Etter en dag med innkubering ble cellene eksponert for fenol. Ved eksponering ble 75 µl medium fjernet fra hver brønn, og 125 µl av ønsket fenolkonsentrasjon tilsatt. Konsentrasjon i brønnene ble 0, 03, 0,1, 0,3, 1, 3, 10 og 30 mM fenol. Etter 48 timers innkubering ble 125 µl igjen fjernet fra brønnene, og125 µl fenol tilsatt. Hemocyttene ble videre innkubert i 48 timer før en viabilitetetstest ble utført (se protokoll i avsnitt 2.1.3.). Viabilitet ble beregnet ved følgende å dividere fluorescens fra eksponert brønn på en tilfeldig valgt verdi fra kontrollgruppen og derfra multiplisert med 100 for å få viabilitet i prosent.

2.1.6. Multixenobiotisk Resistens assay (MXR) – metodeutvikling

Rhodamin 123 eller Rhodamin B evne som substrat, og cyclosporin A, verapamil eller MK 571 evne som hemmer av mekanismen MXR ble testet. Etter 24 timers

innkubering ble 20 µl medium fjernet fra hver brønn, og 10 µl substrat og 10 µl hemmer tilsatt. Ønsket konsentrasjon i hver brønn av substrat var 0,1 µM, og 0,1, 1 eller 5 µM av hemmer. Platene ble satt til innkubering på en orbital ristemaskin i en time på laveste styrke. Etter 1 times innkubering ble cellene vasket to ganger med PBS for å fjerne overskudd av substrat og hemmer. Vaskingen foregikk ved at 100 µl medium ble forsiktig fjernet med en multipipette, og 100 µl ny PBS tilsatt. Etter vaskingen ble alt medium fjernet, og 100 µl 0,1% TritonX-100 (i PBS) ble tilsatt for å lysere cellene. Alt arbeid ble utført i mørke.

Plateleseren ble satt til 535 nm emisjon og 590 nm eksitasjon for Rhodamin B, og 485 nm emisjon og 530 nm eksitasjon for Rhodamin 123. Evne som hemmer ble kvantifisert ved å beregne grad av akkumulert substrat i hemocytter med hemmer i forhold til kontroll (uten hemmer).

2.2 Toksisitetstesting (DEL 2)

2.2.1. Prøvetaking av hemolymfe

Prøvetaking av hemolymfe ble gjort med tidligere beskrevet metode. Hemolymfe fra 30 dyr ble overført i to rør. 15 hunner, gjenkjent av en rød-oransje kappe, i et rør, mens hemolymfe fra 15 hanner, kremgul kappe, utgjorde den andre. Løsningene ble videre uttynnet til 200 000 celler/ ml PBS. 200 µl ble sådd ut i 96 brønners celledyrkningsplater.

2.2.2 *In vitro* eksponering av hemocytter

De bromerte flammehemmerne TBBPA og HBCD og den organiske tinnforbindelsen TBT ble løst i DMSO mens PFOS og PFNA ble løst i aceton. Hemocyttene ble eksponert i 96 timer for stoffene med minst 8 replikater av hver konsentrasjon og kjemikalie. Som kontrollgrupper ble 6 brønner pr brett eksponert for 0,1 % DMSO eller 1 % aceton i PBS. Fenol ble brukt som positiv kontroll, ved konsentrasjonene 0,03, 0,1, 0,3, 1, 3 og 10 mM. Av PFNA ble cellene eksponert for 0,02, 0,2 og 2pM, av PFOS 0,08, 0,8 og 8nM, av TBT 3,16, 31,6 og 316 nM. Av TBBPA og HBCD var eksponeringskonsentrasjonene 5, 10 og 20µM. Eksponeringen foregikk ved at dag 1 ble 75 µl medium fra brønnene fjernet og erstattet med 125 µl eksponeringsløsning. Deretter ble brettene innkubert i 48 timer før re-eksponering. Da ble 125 µl fjernet og erstattet med 125 µl eksponeringsløsning. Cellene ble ytterligere innkubert i 48 timer før videre testing.

2.2.3. Viabilitetstest

Etter 96 timers eksponering ble en viabilitetstetstest utført på de eksponerte cellene med protokoll allerede beskrevet(se protokoll i avsnitt 2.1.3.). Respons på metabolsk aktivitet og membranintegritet hos eksponerte celler ble relatert til tilfeldig valgte verdier hos kontrollgruppen ved å dividere fluorescens fra probene hos de eksponerte gruppene mot kontroll. For å kunne relatere de ulike miljøgiftene til den positive fenolkontrollen ble det laget en fenolekvivalent. Ved å beregne stigningstallet, a, ved fenolstandard for metabolisme, kunne en gjennomsnittelig fenolekvivalent, X, beregnes. Dette ble gjort ved å dividere fluorescens verdi pr eksponerte brønn på a. Fenolekvivalenten viste stoffets tilsvarende konsentrasjon av fenol nødvendig for å oppnå samme toksisk effekt på metabolisme.

2.2.4. MXR test

Etter 96 timers eksponering ble 20 µl medium fjernet fra brønnene, og 10 µl 100 µM cyclosporin A, og 10 µl 2 µM Rhodamin B tilsatt. Halvparten av brønnene ble eksponert for 5 µM cyclosporin A, mens alle brønnene ble eksponert for 0,1 µM rhodamin B eller rhodamn 123. Hemocyttene ble innkubert på en platerister i en time i mørke. Etter innkubering ble cellene vasket to ganger med PBS, og lysert med 0,1 % Triton X-100. Plateleseren ble innstilt på 530-590 nm.
Dataene fulgte ingen normalfordeling så MXR`s grad av hemming/indusering av ble beregnet ved å dividere substratets fluorescens i eksponerte celler på en tilfeldig valgt verdi fra kontrollgruppen og deretter multiplisere med 100. Ved signifikante verdier over 100 hadde en mulig inhibering av MXR funnet sted, og under en mulig induksjon.

2.3. Felt studie i Kastela Bay (DEL 3)

2.3.1. Områdebeskrivelse

Kastela Bay er den største bukta i den sentrale delen av Adriaterhavet med en total overflate på 61 km^2. Utskiftninger av vannet skjer via det smale sundet mellom Brac og fastlandet, og den totale vannfornying skjer i løpet av en måned. Området er kjent som et av de mest forurensede områdene i Adriaterhavet med byen Split som er den nest største byen i Kroatia og en mengde bosettinger rundt bukta. Ubehandlet eller delvis ubehandlet urbant avfall og industrielt avfall slippes direkte ut. Hovedindustrier langs bukta er skipsverft, sementproduksjon, kjemiske fabrikker og metallindustri. I Kastela og Trogir er kun 25% av befolkningen tilknyttet vann og kloakk (Margeta, 2001). Etter kjemiske analyser i 2003 utført av NIVA (tabell 1) ble store mengder PAHer, PCBer og tungmetaller funnet i blåskjell i dette området (Anders Ruus, personlig komment).

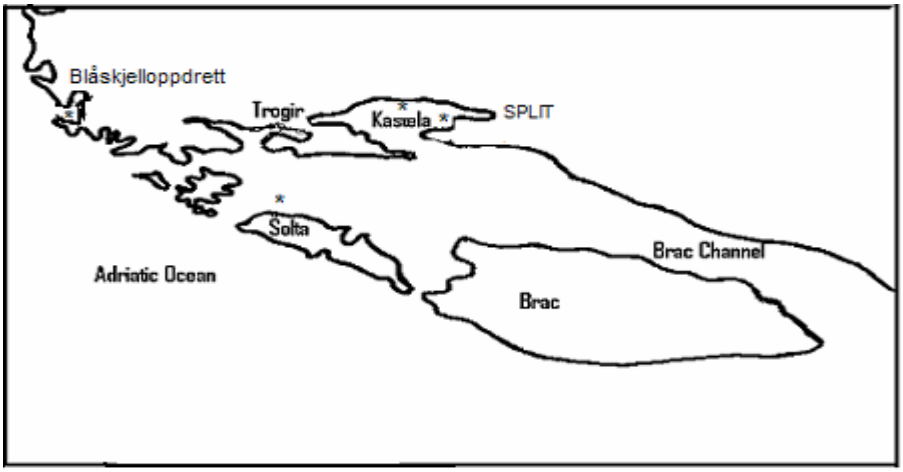
Tabell 1. Resultat etter kjemiske analyser av *M. Galloprovincialis* i 2003.

Stoff (µg/g dw)	Vranjic	Adriavinil	Trogir
Hg	0,011	0,4	0,017
Cd	0,043	0,092	0,128
Cu	1,2	1,29	2,9
Zn	24	17,9	32,8
PCB	26,27	25,69	92,26
PAH	118,4	-	-

2.3.2. Innsamling av blåskjell

M. galloprovincialis ble samlet inn ved fem ulike stasjoner ulike på buktens innside og utside (se figur 7). Ti dyr ble prøvetatt fra hver stasjon. Øya Šolta, som var antatt ”fri” for kontaminering, utenfor Kaštela bukta ble valgt som referansestasjon. Adriavinil lå i nærheten av en gammel fabrikk og hadde antatte utslipp av kvikksølv. Vranjic dekket Split`s hovedkloakkutslipp. Ved skipsverftet i Trogir ble kun fem dyr samlet inn på grunn av høy dødelighet i de utsatte burene. Fra en blåskjellfarm utenfor bukta ble 10 dyr prøvetatt. Det er sannsynlig å tro at dyrene har vært eksponert for store mengder miljøgifter som blant annet kvikksølv og bly, antibegroingsmidler, legemidler og halogenerte hydrokarboner. Dyrenes størrelse

varierte fra 4-8 cm, og ble oppbevart i 100 liters plastdunker med rent ventilert sjøvann over natten før prøvetaking neste dag.



Figur 7. Tredimensjonalt kart over Kaštela Bukta. Innsamlingsområder for blåskjell er merket med stjerne.

2.3.3. Prøvetaking av hemolymfe

Hemolymfe ble tappet fra bakre lukkemuskel med en 1 ml kanyle og 23 gauge sprøytespiss forvasket med kald PBS 36 ‰ NaCl. Hemolymfen ble overført til individuelle glasstuber som inneholdt 1 ml PBS. Prøvene ble ytterligere fortynnet ved å tilsette en lik mengde PBS som volumet i tubene. 200 µl av hvert individ ble sådd ut på 96 brønners celledyrkningsbrett. Brettene ble innkubert over natten.

2.3.4. Kvantifisering av celletall

Av utynnet hemolymfe ble 50 µl fiksert i 150 µl paraformaldehyd og lagret i 1,5 ml eppendorf rør. Kvantifisering av hemocytter ble gjort med Multisizer^{TM} 3 Coulter counter ved prosedyrer allerede beskrevet. Konsentrasjon ble videre beregnet ved dividering av fiksert volum på 1/4.

2.3.5. Viabilitetstest

Dag tre ble en viabilitetstest utført med prosedyrer allerede beskrevet. Dataene var ikke normalfordelte og viabilitet av hemocytter ble beregnet ved følgende formel:

Viabilitet= fluorescens $_{prøve}$/tilfeldig fluorescens $_{referanse}$

2.3.6. Multixenobiotisk resistens test

Fra hver brønn ble 20 µl medium fjernet og erstattet med fluoriserende 20 µl 50 µM rhodamin 123. Etter 1 times innkubering ble cellene vasket to ganger før lysering med 100 µl 0,1% Triton X-100. Plateleseren Fluorlite 1000, Dynatech, hadde filter med eksitasjon og emisjon 485-530. MXR aktivitet ble relatert til akkumulert rhodamin 123 i hemocytter fra dyr innsamlet ved Solta.

MXR aktivitet= (fluorescens $_{prøve}$/tilfeldig fluorescens $_{referanse}$)

2.4. Databehandling

Data ble presentert i ”box-plot”. Boksene viser median og kvartiler (25 og 75 persentiler), mens ”whiskers” viser 10 og 90 persentiler. Uteliggere og ekstrempunkter ble fjernet, og defineres av programvaren etter følgende krav:

Uteliggere;

datapunktverdier > UBV + o.c.(UBV - LBV)*

d*atapunktverdier < LBV - o.c.*(UBV* - LBV)

Ekstremverdier defineres ved disse kravene.

*datapunktverdier > UBV + 2*o.c.*(UBV - LBV)*

*datapunktverdier < LBV - 2*o.c.*(UBV – LBV)*

hvor UBV er øvre verdi av boksen i boksplottet, LBV er nedre verdi i boksen og o.c. er uteligger koeffisienten 1.5 (StatSoft, 2001).

For å sammenligne ulike grupper ble det benyttet enveis-variansanalyse (ANOVA) og Levenes test ble benyttet for å teste homogen varians (Lim & Loh, 1996). Ved $p \geq 0.05$ ble Levenes test forkastet, og enveis-ANOVA brukt. Med signifikans $p \leq 0.05$ ble hypotesen om homogen varians avvist, og den ikke parametriske testen Kruskal-Wallis brukt (Lowry, 2005; StatSoft, 2001). Ved homogen varians ble det benyttet enveis- ANOVA.
Signifikans ble angitt med $p \leq 0{,}05/n$ jamfør Bonferroni korreksjon. Ved *in vitro* test var n=3, og i feltstudie n=4.

H_0: Ingen forskjell mellom eksponerte celler og kontroll celler.
H_0: Ingen forskjell mellomreferansestasjon og innsamlingsområde.
Signifikant nivå for forkasting av H_0 ble satt til 0,05/n, der n er antall gjentatte tester med samme gruppe.

Programvaren som ble brukt var Statistica 6.0 fra StatSoft corporation.

Del 1. Utvikling av metode

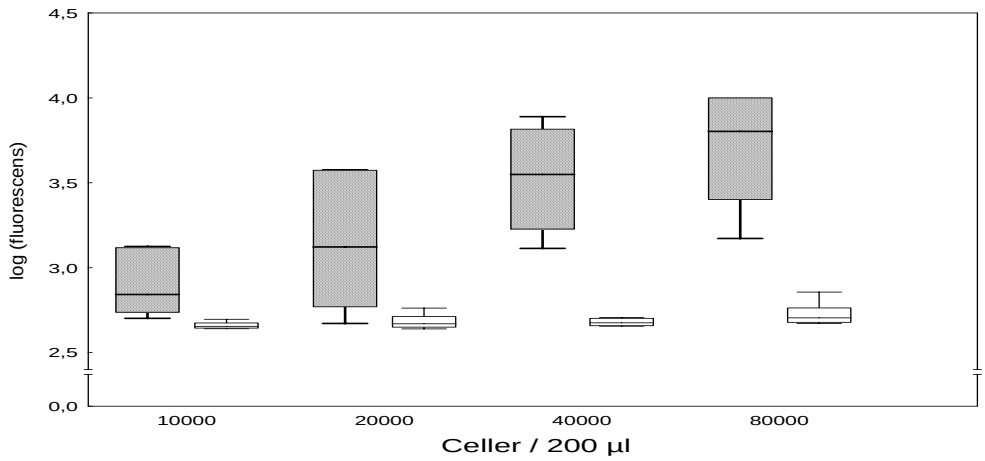
Etablering av metode for testing av cytotoksisitet og av multixenobiotic resistens

3. Resultater

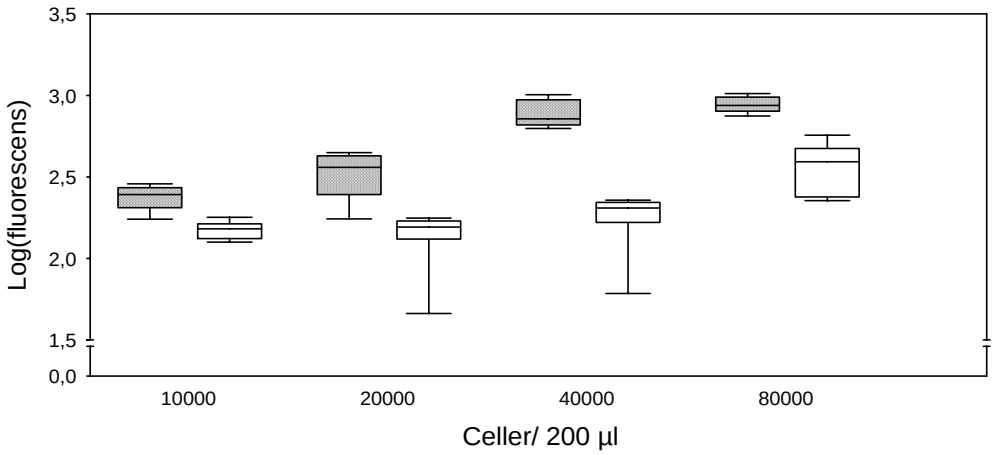
3.1 Etablering av metode for testing av cytotoksisitet og av multixenobiotic resistens

3.1.1. Medium og celletetthet

Ved metodeetablering ble PBS og L-15 testet som medium, og optimalisering av cellekonsentrasjon utført med konsentrasjonene 10 000, 20 000, 40 000 eller 80 000 celler per brønn. Det var signifikante forskjeller på hemocytter dyrket i de to mediene. Celler dyrket i PBS viste størst stigende metabolsk aktivitet (se figur 8) og høyest membranintegritet (se figur 9). Membranintegriteten hadde en signifikant økning ved økende celletetthet med utflating av kurven mot 80 000 celler pr brønn. Det var ingen signifikante forskjeller mellom 40 000 celler eller 80 000 celler pr brønn. Metabolismen steg også i korrelasjon med celletetthet.



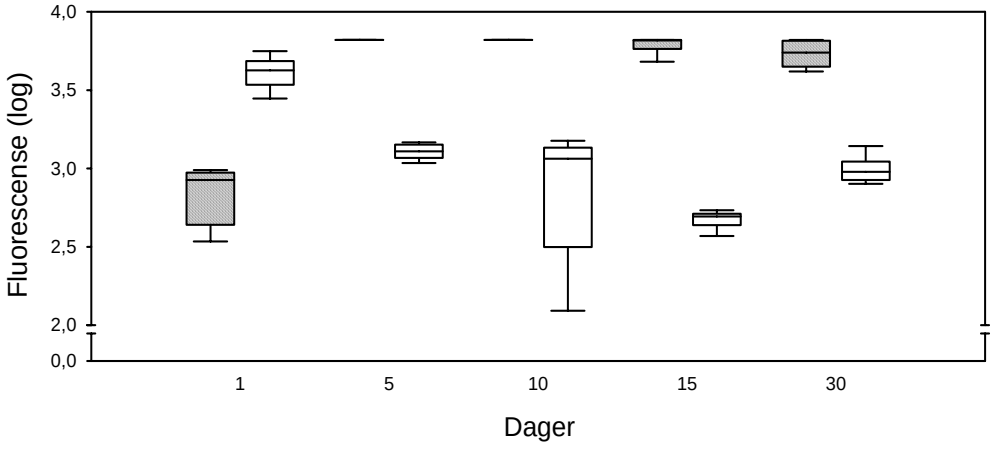
Figur 8. L-15 eller PBS som cellemedium og deres effekt på metabolsk aktivitet. PBS-skraverte bokser. L-15-hvite bokser. 16 replikater. Y- aksen har et skalabrudd fra 0,2 til 2,4. Boksene viser middeldverdi og kvartil og whiskers viser 10 og 90 persentil.



Figur 9. L-15 eller PBS som cellemedium og deres effekt på membranintegritet. PBS-skraverte bokser. L-15-hvite bokser. 16 replikater. Y- aksen har et skalabrudd fra 0,2 til 1,5. Boksene viser middeldverdi og kvartil og whiskers viser 10 og 90 persentil.

3.1.2. Hemocytters viabilitet ved ulike tidspunkter

I primærkulturen bestående av hemocytter og PBS ble endring av metabolisme og membranintegritet målt ved dag 1, 5, 10, 15 og 30 (se figur 10). Metabolsk aktivitet steg fra dag 1 mot neste måling dag 5. Mellom dag 5 og 15 var det ingen signifikante forskjeller. Dag 30 var det en minking i metabolsk aktivitet fra dag 5, 10 og 155, men fremdels betydelig høyere enn dag 1. Membranintegritet hadde ved dag 1 en topp for deretter å minke signifikant mot neste måling dag 5. Dag 5, 10 og 30 viste ingen signifikante endringer, men dag 15 var signifikant lavere.



Figur 10. Endring av viabilitet på ulike tidspunkter. Skraverte bokser- metabolsk aktivitet (alamar Blue). Åpne bokser- membranintegritet (CFDA-AM). Skalabrudd på Y-aksen fra 0,2 – 2. Boksene viser median og kvartiler og whiskers 10 og 90 persentil.

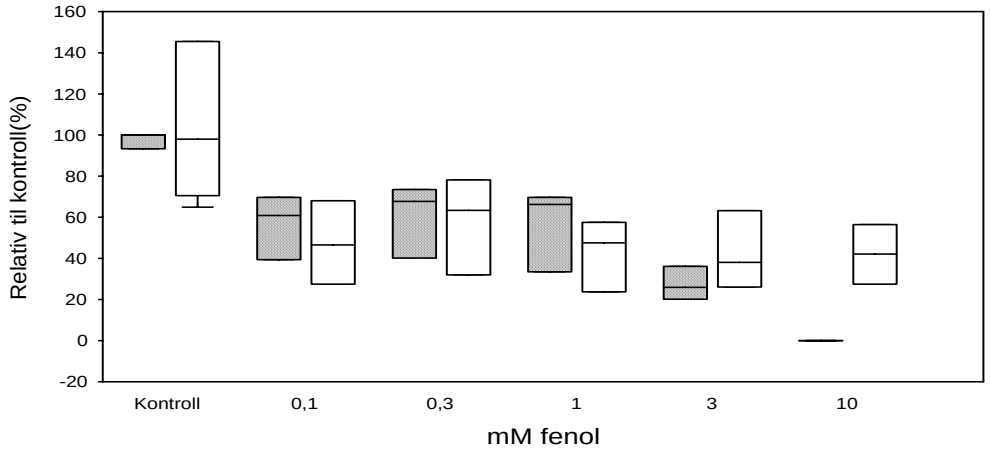
3.1.3. Fenol som standard

Metabolsk aktivitet og membranintegritet i hemocytter eksponert for 0,1, 0,3, 1, 3 og 10 mM fenol er utrykt relativt til % ut ifra tilfeldig valgte verdier hos kontrollgruppen. Metabolske aktiviteten sank signifikant (p=0.01, Enveis-ANOVA) fra kontroll mot hemocytter eksponert for 10 mM fenol (se fig 11). Det var ingen signifikante forskjeller mellom 0.1, 0.3 og 1 mM fenol hvor median aktivitet var 60% sammenlignet med kontroll. Ved mM fenol lå median på 26% og ved 10 mM var metabolsk aktivitet 0. Membranintegritet sank signifikant (p=0.000147, Enveis-ANOVA) fra kontroll til 0,1mM fenol med 50%. Mellom 0,1 og 10 mM fenol var det ingen signifikante endringer. Fenolekvivalent for metabolsk aktivitet ble beregnet ved å foreta en regresjonsanalyse på metabolsk aktivitet. Stigningstallet, a, ble videre brukt til beregning av fenolekvivalent.

Fenolekvivalent =aX +100% metabolisme i kontroll

Hvor a er –10 (stignigstallet) og X er mM fenol.

EC50 for membranintegritet ble beregnet til 0.1 mM fenol, og for metabolisme ble EC50 beregnet til 5 mM fenol.

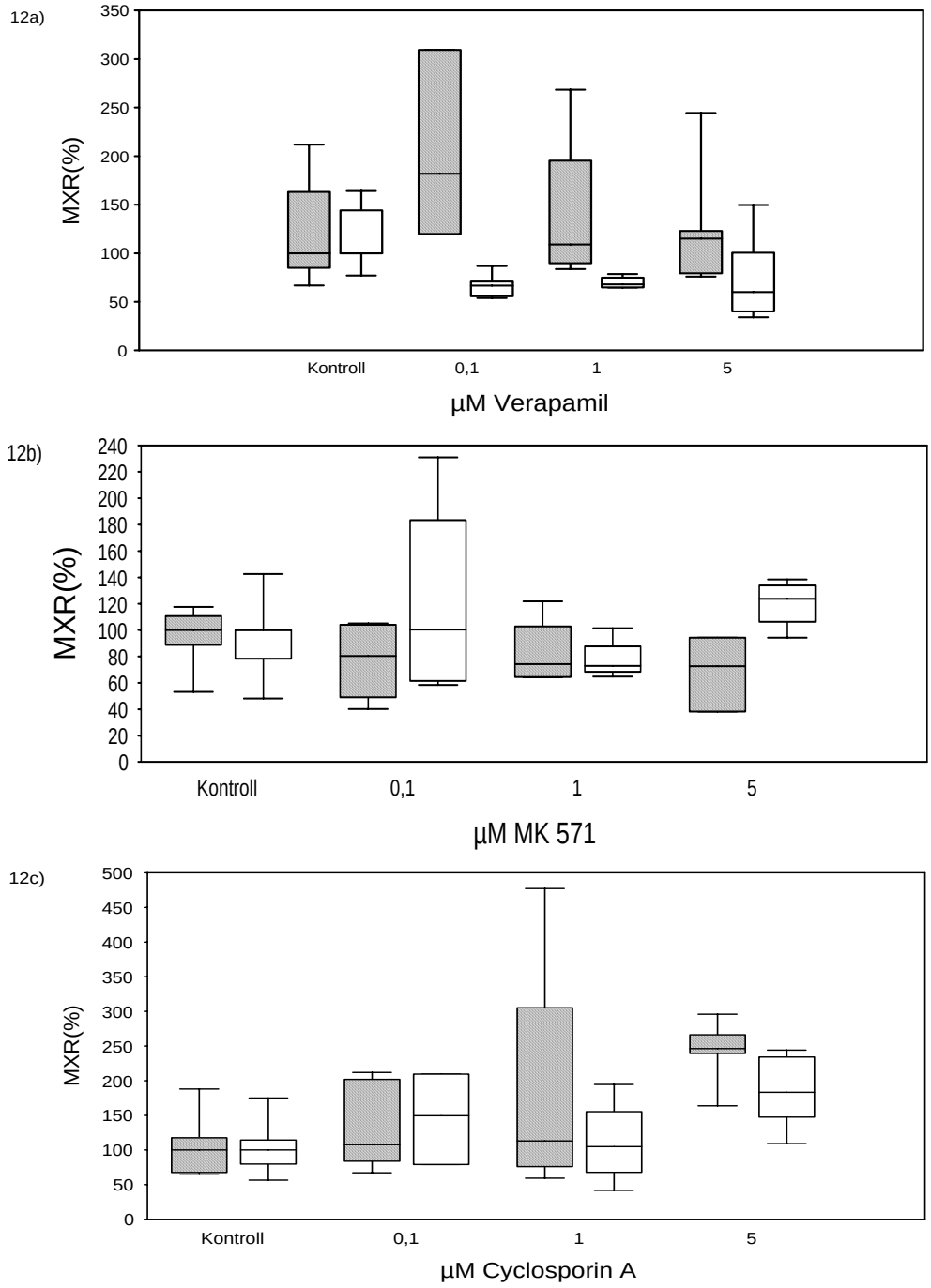


Figur 11. Etablering av standardkurve for testing av cytotoksisitet. Skraverte bokser- alamar Blue (metabolsk aktivitet). Åpne bokser- CFDA-AM (membranintegritet). Linjer gir median og kvartiler, whiskers er 10 og 90 persentil.

3.1.4. Etablering av multixenobiotisk resistens assay, MXR

MXR aktivitet i cellene ble relatert til prosentvis akkumulering i hemocytter av substratet (Rhodamin 123 eller Rhodamin B) hemmet med MK 571, verapamil eller cyclosporin A i forhold til kontroll (kun substrat og hemocytter). Ved bruk av 0.1, 1 eller 5 µM Verapamil og MK 571 skjedde det ingen hemming av MXR aktivitet (se

figur 12a og b). Cyclosporin A (se figur 12c) som hemmer og 0,1 µM rhodamin 123 som substrat ga ingen signifikante forskjeller på hemocytter behandlet med 0,1, 1 eller 5µM. Med substratet Rhodamin B var det en signifikant økning på 250 % (p= 0.0165, Kruskal-Wallis) ved 5µM cyclosporin A. 0,1 og 1µM cyclosporin A ga ingen signifikant økning av Rhodamin B i cellene mot kontroll.



Figur 12. Uttesting av substrat og hemmer ved etablering av MXR test for hemocytter. Skraverte bokser- fluorescens Rhodamin B, åpne bokser- fluorescens Rhodamin 123. Det er 8 replikater pr konsentrasjon. Linjer viser median og kvartil, whiskers 10 og 90 persentil.

4. Diskusjon – metode- etablering

4.1. Celletetthet og medium

PBS og L-15 som cellemedium ga høyest membranintegritet og metabolsk aktivitet ved bruk av PBS. L-15 ga lavere metabolisk aktivitet og senket membranintegritet. Ut ifra dette kan det virke som at dette mediet er toksisk for hemocytter. Et annet studie utført på hemocytter har vist at tilsetting av antisoppmidler virket cytotoksisk på cellene (Cao et al., 2003). I L-15 tilsatte vi antifungicon, og dermed kan dette ha vært årsaken til L-15 senkede viabilitet hos hemocytter. En annen mulighet kan være kompleksdannelser mellom prober og medium som hindrer videre redusering til produkt som avgir fluorescens. Dette, og mulig toksisk effekt utelukker bruk av L-15 som medium ved de betingelser fulgt i denne oppgaven.

Den metabolske aktiviteten steg i korrelasjon med cellekonsentrasjon ved høyest fluormetrisk utbytte av proben alamar Blue ved 80 000 celler pr brønn, mens det var ingen forskjeller mellom 40 000 og 80 000 celler og membranintegritet. Dette kan skyldes at proben CFDA-AM var begrensende ved 40 000 celler, og dermed forhindret videre omdannelse til fluorescens, eller at 80 000 celler ble for høy tetthet slik at cellene ikke fikk feste seg og dermed fjernet ved tilsetting av probene. En annen mulighet er stress ved for høye konsentrasjoner som senker cellenes overlevelse. Metabolisk aktivitet og membranintegritet sett i sammenheng kan tyde på at 40 000 celler pr brønn er passende konsentrasjon, og at CFDA-AM er den begrensende faktor ved mål på membranintegritet

4.2. Endring av viabilitet over tid

Hemocytters evne til å holde seg levedyktige over lengre tid viste seg å være stor. Membranintegritet sank fra dag 1, men holdt seg stabil over lengre tid. Først etter 15 dager kunne man se endring i metabolsk aktivitet i mitokondriene, og dag 30 var cellene fremdeles viable. Senket integritet ved dag 15 kan skyldes mindre viable celler. Andre studier viser ingen endring i viabilitet før dag 20 ved bruk av L-15 uten antisoppmiddel (Domart-Coulon et al., 1994). L-15 virket toksisk på cellene og egner seg ikke som medium ved de betingelser fulgt i denne uttestingen. Andre studier har vist at tilsetting av antisoppmidler har virket svært cytotoksisk på hemocytter (Cao et al., 2003). Disse resultatene antyder at hos hemocytter fra

Mytilus edulis kan holdes i kultur over lengre tid uten at metabolsk aktivitet og membranintegritet hemmes betraktelig.

4.3. Etablering av standardkurve ved viabilitetstesting

Ved etablering av standardkurve for videre viabilitetstesting ble metabolsk aktivitet og membranintegritet testet. Metabolsk aktivitet viste en synkende dose respons kurve ved de testede konsentrasjoner av fenol mot 10 mM fenol, mens membranintegritet sank fra kontroll mot 0,1mM fenol. Etter dette var det ingen endring av integritet. Metabolsk aktivitet viser seg å være sensitiv for fenol, og dermed egnet som positiv kontroll av andre stoffers effekt. Membranintegritet er ikke like sensitiv ved de testede konsentrasjoner av fenol. Metabolisme i en celle er svært viktig for dens overlevelse, og dermed også interessant i et økotoksikologisk perspektiv. Relatering til fenol konsentrasjon og metabolsk respons av andre stoffer kan derfor være meget nyttig, og vil bli gjort i denne oppgaven. Membranintegritet er også en viktig markør for hemocytters viabilitet, men videre forskning må til for å finne et egnet stoff som standard for både metabolisme og membranintegritet for å kunne gjøre et slikt assay så enkelt og billig som mulig.

4.4. Etablering av multixenobiotisk resistens test, MXR.

Ved de konsentrasjoner av substrat og inhibitorer testet var det 0,1µM rhodamin B og 5µM Cyclosporin A som ga størst akkumulering av substrat, og dermed også hemming av MXR-aktivitet. Rhodamin 123 har blitt vist i andre celler å virke som modellsubstrat (Egudina et al., 1993; Wang et al., 2004), men ga ingen resultater i dette forsøket. Verapamil og MK 571 er vist å virke som modellsubstrat og hemmer for MRP1 og MDR1 proteiner celler hos pattedyr (Hong et al., 2003; Keppler et al., 1996) og hos akvatiske organismer (Domart-Coulon et al., 1994; Kurelec, 1995; Kurelec & Pivcevic, 1991; Minier & Moore, 1996; Wu et al., 2005; Wu et al., 2005), men i dette forsøket ga det ingen akkumulering. Som substrat MXR assay vil Rhodamin B brukt, og som hemmer vil cyclosporin A brukt videre i denne oppgaven.

Konklusjon metodeutvikling

Ved denne metodeutviklingen har det blitt vist at hemocytter fra M. edulis kan holdes i en primær cellekultur med PBS som medium. Bruk av 40 000 hemocytter (200 000 /ml) var passende konsentrasjon pr brønn, og de holder seg viable over en lengre tidsperiode. Hemocyttene lar seg bruke i toksisitetstester med MXR aktivitet og celleviabilitet som parameter. Ved videre arbeid med hemocytter og cellekultur ble PBS og 40 000 celler pr brønn brukt. Fenol vil bli brukt som standard ved metabolsk aktivitet og cyclosporin A som hemmer og Rhodamin B som substrat ved MXR aktivitet.

Del 2. In vitro toksisitetstest

The use of *Mytilus edulis* hemocytes to test the *in vitro* cytotoxicity of environmental contaminants.

The use of *Mytilus edulis* hemocytes to test the *in vitro* cytotoxicity of environmental contaminants.

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Abstract

Within biomonitoring it has been common practice to analyse the concentration of pollutants in organisms. However, focus is shifting towards an approach which combines chemical analyses with measurements of effects in organisms after exposure to contaminant. In this study, the objective was to test developed methods in pilot studies conducted earlier. One of the aims was to test whether hemocytes from the blue mussel Mytilus edulis *could be used in* vitro toxicity *testing with levels of contaminants not far from concentration found in marine biota. Bioassays to asses cell viability and multixenobiotic resistance activity after exposure to the environmental contaminants HBCD, PFNA, PFOS, TBT and TBBPA were conducted. A second aim was to clarify whether there are differences between hemocytes sampled from male and female blue mussels with regard to exposure of contaminant. The hemocyte viability decreased in both male and female mussels after 96 hours exposure to 5, 10 and 20 µM of the brominated flame retardants TBBPA and HBCD and 3.16, 31.6 and 316 nM TBT. The brominated flame retardants had the strongest effect on membrane while TBT showed a stronger effect on metabolic activity. In male hemocytes there was a significant inhibition of MXR activity at 316 nM TBT. The hemocyte viability was not decreased after exposure to PFOS and PFNA, but this contaminants were strong inhibitors of MXR activity. Female hemocytes seemed to have a higher degree of MXR inhibition than hemocytes sampled from male mussels. The same results after exposure to 0.02, 0.2 and 2 pM PFNA were found. Viability in male hemocytes seemed to be more affected after exposure to HBCD, TBBPA and TBT, while female hemocytes seemed to possess a higher MXR activity showing a higher degree of MXR inhibition. With higher levels of MXR activity the accumulation of xenobiotics might be prevented, resulting in a decreased cytotoxicity of the accumulated contaminants. Hemocytes as biomarkers with cell viability and MXR activity as endpoints could be successfully used as they react to low concentrations of contaminants.*

Introduction

The use of mussels in toxicity testing has several advantages. Bivalves are dominant members of coastal and estuarine communities and have a widespread geographical distribution. They are sedentary and relative tolerant to a wide range of environmental conditions. Mussels are also filter feeders that pump large volumes of water and concentrate pollutant in their tissues and make it available to other trophic levels in the environment as an important part of food supply among aquatic animals (Dane, 1996; Widdows & Donkin, 1992). The hemolymph system in bivalves is an open circulation system with hemolymph containing hemocytes in sinus bathing the tissues directly. Hemocytes are not confined only to the hemolymph system, but moves freely in and out of the sinus into surroundings connective tissues, mantle cavity and gut lumen, and represent the most important internal defense mechanism in marine bivalves (Gosling, 2003). The macrophage reacts to foreign substances by phagocytosis or encapsulation. The process involves several stages: non-self recognition, cell proliferation, locomotion, binding and digestion of particle matter and intracellular degradation particles (Hill & Welsh, 1966; Martins et al., 2005). Hemolymph plays also an important role in gas exchange, osmotic regulation, nutrient distribution, elimination of wastes and internal defense. Studies conducted earlier indicate that contaminants might have an unfavorable effect on the immune system (Bihari et al., 2003; Canesi et al., 2005; Canesi et al., 2002; Luengen et al., 2004; Martins et al., 2005; Oliver et al., 2003).

In the field of biomonitoring chemical analysis of water samples and animal tissues has played a major role. Nevertheless, knowledge about a chemical potential to cause injurious effects after chemical analysis is limited. A lot of the substances present cannot be detected because number of chemicals in the sample are large, the effect on a chemical mixture working in is hard to predict and many pollutant are undefined degradation products or micropollutants (Tonkes et al., 2005). The external levels of exposure and internal level of tissue contamination and early adverse effect has to be established to get a connection between exposure in water and level of injury. To establish such connections the common practice has been determination of lethal concentrations, LC50 values. Common procedures has been to expose a group of individuals to different concentrations, and then determine at

which concentration there is a 50% mortality (Eaton & Klaasen, 2001). Today, the practice is shifting towards determination at effects on cellular or tissue levels rather individual level (van der Oost et al., 2003).

Bioassays have the advantage of directly measuring the toxic effect of bio-available substances on aquatic organisms, and they measure both known and unknown hazardous substances (Tonkes et al., 2005). Assay of cell viability have a broad application both within cell culturing and in the use of *in vitro* toxicology. In this experiment we will use the two molecular probes alamar Blue™ and CFDA-AM as measurement of membrane integrity and metabolic activity respectively. Alamar Blue™ is reduced by cellular reductases, possibly by diaphorases or by NADH dehydrogenase (O'Brien et al., 2000). The reduction has been believed to take place on the mitochondria of the cells, but no evidence for this was found by O'Brien, Wilson et al. (2000). In this article, alamar Blue™ fluorescence will be referred to as metabolic activity. The esterase activity is measured by the molecular probe 5-carboxyfluorescein diacetate, acetoxymethyl ester, CFDA-AM. The substrate diffuses rapidly into the cell while the product diffuses slowly out of the cells and fluorescence readings will reflect the membrane integrity of the cells. Because the two probes are measured at different emission wavelengths both dyes can be added together to perform the assay in one single step, and provide a measure for cell viability (Ganassin et al., 2000).

It has been shown that aquatic organisms possess a mechanism similar to the multidrug resistance phenomenon observed in tumour cells resistant to anti-cancer drugs (Keppler & Ringwood, 2001; Kurelec, 1992; Kurelec & Pivcevic, 1991; Minier & Galgani, 1995). Induction of this mechanism, named multixenobiotic resistance (MXR), has been reported in numerous studies of aquatic organism after exposure in laboratory or in environment by anthropogenic contaminants or natural stress (Eufemia & Epel, 2000; Kurelec et al., 1995; Minier et al., 2000; Minier & Moore, 1996; Smital et al., 2003). MXR is caused by energy-dependent pumping of substances out of the cells. Substrates are both endogenous chemicals and xenobiotics, thus preventing their accumulation and toxic effect (Kurelec, 1992). The protective role of the defence appears to be fragile: as opposed to MXR induction, it is also demonstrated that there are many classes of chemicals which are

capable of inhibiting the MXR function. These are referred to as chemosensitisers, and can be environmentally hazardous chemicals, because they may lead to accumulation of xenobiotics and elevate internal levels of toxins (Smital & Kurelec, 1998). Studies conducted have shown that there are several different groups of chemicals which are inhibiting the MXR mechanism. These agents seems to have in common a carbonyl group and to act hydrophobic (Kurelec, 1992). A method to measure the activity is adding a known fluorescent MDR/MXR substrate and a known inhibitor. The accumulated substrate in inhibited cells compared with a control could be used as a measurement on MXR activity (Kurelec, 1995).

The two previous described methods were used in this work to test the cytotoxic effect and property as MXR inhibitors or inductors of five different contaminants. The contaminant had in common that they are on the Norwegian government list on possible hazardous chemicals with regard to human health and environment. These chemicals are widely used and have been detected in human, terrestrial and aquatic samples. They are persistent, are shown to accumulate in organisms, might have severe chronic effect or be highly toxic to the environment (SFT, 2004).

Brominated flame retardants are substances used in plastics, textiles, electronic circuitry and other materials to prevent fires. Knowledge is very limited and hinders environmental authorities from carrying out environmental risk assessments (de Wit, 2002). From the group of brominated flame retardants, hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) where chosen. The total use in 2002 was between 280-330 ton (SFT, 2005) in Norway. They are shown to interact with the thyroid hormone system endocrine and estrogenic system in mammals (Legler & Brouwer, 2003) and induce oxidative stress *in vivo* in rainbow trout with an inhibition of EROD (Ronisz et al., 2004).

Perflourinated chemicals are another group of environmental contaminants on the list. As they are difficult to degrade by acid, alkaline or heat they are commonly used in foam put in fire extinguisher, fabrics and paper. They are not yet forbidden in use by the Norwegian government, but as they has been shown to easily accumulate in fat and to cause reproductive toxicity during breast feeding in rat they are considered as potential hazardous chemicals. Release into the environment was in 2004 estimated to be 12-15 ton.

Organic tin compounds are have been shown to bind to biological molecules, interact with cell membranes, change the ionic balance *in vivo* and impair mitochondria by blocking of the ATP syntase (Huang & Wang, 1995). They have been widely used as antifouling, in painting, as disinfectant and as preserving agent, and they are often found in high concentration around shipbuilding yards, marinas, and harbours ore close to such places. In mussels and whelks there are found elevated levels of tributyltin. Despite the fact that TBT is not allowed used on boats smaller than 25m, content in mussels and whelks monitored has not changed since 1997 along the Norwegian coastal zone. High degree of imposex in marine whelks has been shown to correlate with degree of TBT concentration. New regulation has forbidden all use of TBT after 2007. Release of TBT into the Norwegian environment has been reduced from 58 ton in 1985 to 7 ton in 2003 (SFT, 2005).

The aims of this article were to test the sensitivity of hemocytes from the blue mussel *Mytilus edulis* to some selected environmental contaminants, using cytotoxicity and MXR as endpoints. Further to clarify whether there are differences between hemocytes sampled from male and female blue mussels.

Materials and methods

Animals

Mussels were collected during June from Norwegian Institute for Water marine research station at Solbergstrand outside of Drøbak at the inner part of the Oslo fjord. The temperature of the water was holding 17˚C, and the salinity of the water was 24‰S. Fifteen animals of each sex were collected and distinguished by the color of their mantle. Male *M. edulis* were recognised by a creamed-colour and the females by reddish. The organisms were kept in a plastic box during sampling of hemolymph.

Collection of hemolymph

Hemocytes were collected by the withdrawal of hemolymph from their adductor muscle using a 23-gauge needle and 1 ml syringe pretreated with cold PBS 24‰NaCl. Hemocyte counts were recorded using a Multisizer^{TM} 3, Coulter counter. The hemocytes were further diluted with cold PBS to hold a concentration of 200 000 cells /ml. To each well in a 96 wells microplate 200 µl of the suspensions were

seeded. The micro plates were put in an incubation chamber over night to let the hemocytes attach to the walls.

In vitro exposure of hemocytes

The brominated flame retardants TBBPA, and HBCD were solved in DMSO (Chiron, 2005), PFOS and PFNA were solved in acetone and TBT (Sigma-Aldrich, 2005) was solved in DMSO. The hemocytes were exposed for 96 hours to eight replicates of each concentration and pollutant, eight blank wells with only PBS and control groups with 0,1% DMSO (in PBS) or 1% acetone (in PBS). The concentrations of pollutants were 0.02, 0.2 and 2 pM PFNA; 0.08, 0.8 and 8 nM PFOS; 5, 10 and 20 µM TBBPA and HBCD, and 3.16, 31.6 and 316 nM TBT. As positive control to the cytotoxicity assay a phenol standard of 0.03, 0.1, 0.3, 1, 3 and 10mM phenol was made up in DMSO. After adding the pollutants to the wells the hemocytes were incubated in 48 hours before re-exposure.

Cytotoxicity assay

After 96 hours of exposure a cytotoxicity assay was carried out to the exposed cells. The medium was carefully removed from the wells, and 100 µl of a mixture consisting of 11.6 µl 4 mM CFDA-AM (Molecular Probes), 579 µl alamar Blue ^{TM} (Biosource) and 11 ml PBS was added to each well, and put on an orbital table shaker for 30 minutes. All work was done in the dark to avoid daylight illumination. The fluorescence plate reader was set to excitation and emission wavelength pairs of 530-590nm (alamar Blue) and 485-530nm (CFDA-AM). After conducting readings the average of the blank wells were calculated and subtracted from data. The effect on integrity of mitochondria metabolism and membrane was related to the control by dividing the fluorescence in hemocytes exposed to contaminants with a random chosen fluorescence value in control group as the data did not follow normal distribution. To calculate the equivalent concentration of phenol needed to get the same effect on metabolic activity a regression analysis were made and a factor calculated, a, which gave the slope of the curve. In female hemocytes; a is equal –10, and male; equal to –8. From this analysis following formulae were made:

$$Phenol\ equivalent = (Viability_{pollutant} - random_{control})/a$$

MXR assay

Twenty µl medium was removed from the wells after the exposure and replaced with10 µl 100 µM cyclosporine A as inhibitor, and 10 µl 2 µM Rhodamine B as substrate. Half of the wells were exposed to substrate and inhibitor and the other half were only exposed to the substrate. The hemocytes were incubated on an orbital table shaker in dark for 1 hour. After incubation the hemocytes were washed twice with PBS to remove excess substrate. Then medium were removed and 100 µl 0.1 % Triton X-100 were added to make the hemocyte membrane burst. The plate reader was set to emission 530 and excitation 590 nm. From a random value of the control fluorescence of Rhodamine B the pollutants property as MXR inhibitors were calculated.

$$Inhibiton/induction\ MXR = (Fluorescence_{pollutant}/random\ fluorescence_{control})*100$$

MXR > 100: Possible inhibition of MXR mechanism
MXR < 100: Possible induction of MXR mechanism

Statistical procedures

To test whether or not hemocytes exposed to contaminants were different from the control group one-way analysis of variance(ANOVA) and Levene`s test for testing the homogeneity of variance were used (Lim & Loh, 1996). With $p \geq 0.05$ and homogeny variance one-way ANOVA was used. With $p \leq 0.05$ the non-parametric test Kruskal-Wallis was used (Lowry, 2005; StatSoft, 2001). Significance was set to $p \leq 0{,}05/n$, $p \leq 0.016$ according to Bonferroni correction(Fisher & van Belle, 1993; Roth, 1999). In this article n=3.

Results

Exposure to phenol

In the hemocytes sampled from female *M. edulis*, and exposed to phenol there was a significant correlation with increasing concentration of phenol and decreasing metabolic activity. The hemocytes seemed to be unaffected at 1 mM phenol (figure 1.a). At 10 mM the activity was not measurable with the probes, and at 3 mM the activity was at 60% compared to control (significant with p=0.01). EC50 was

calculated to be 5 mM phenol. The membrane integrity did not show any significant difference at the tested concentrations of phenol.

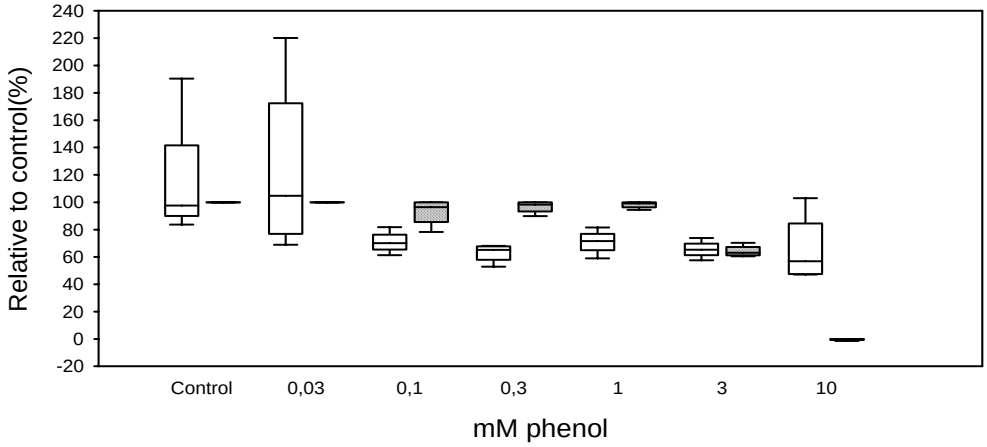

Figure 1a) Effects of phenol on female mussel hemocytes. Shaded-alamar Blue (metabolic activity; open-CFDA-AM (membrane integrity). Measurements in triplicate; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

In hemocytes sampled from male *M. edulis* the metabolic activity was significant different from control (p=0.01, oneway-ANOVA). The hemocytes seemed to be unaffected at 0.3 mM phenol with regard metabolic activity. At 3 mM the median activity was 40% of the control group median, and at 10 mM there was no measurable activity (figure 1.b). EC50 of phenol in male hemocytes was calculated to be 4.1 mM. The membrane integrity did not show ay significant difference at the tested concentrations of phenol.

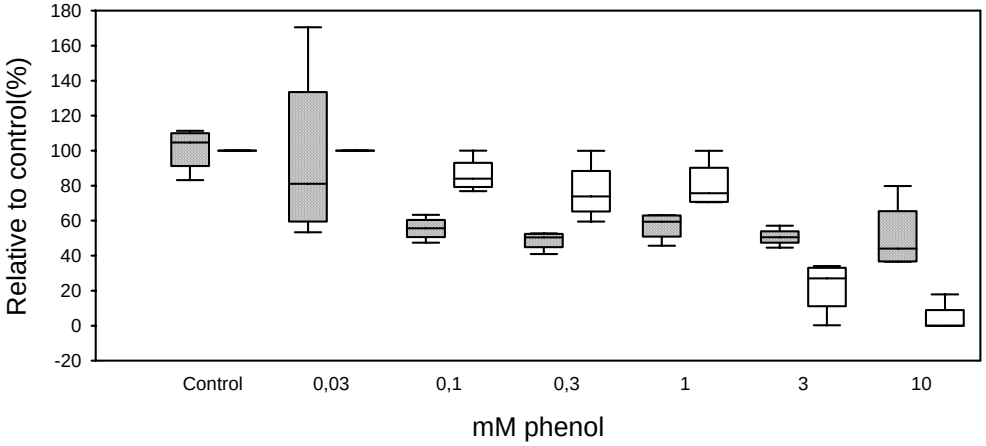

Figure1.b) Effects of phenol on male mussels hemocytes. Shaded - alamar Blue (metabolic activity); open - CFDA-AM (membrane integrity). Measurements in triplicate; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

MXR inhibition by cyclosporine A

After 1 hours exposure with inhibitor cyclosporine A the female hemocytes had a higher accumulation of substrate than male hemocytes (figure 2). Hemocytes from female mussels had a significant increase in accumulation (p=0.001, Kruskal-

Wallis). Median concentration of substrate in female hemocytes inhibited with 5 µM cyclosporine A was 188%. In male hemocytes there was no significant accumulation or decrease after 1 hour exposure to inhibitor. The accumulation was higher than control with median =131%.

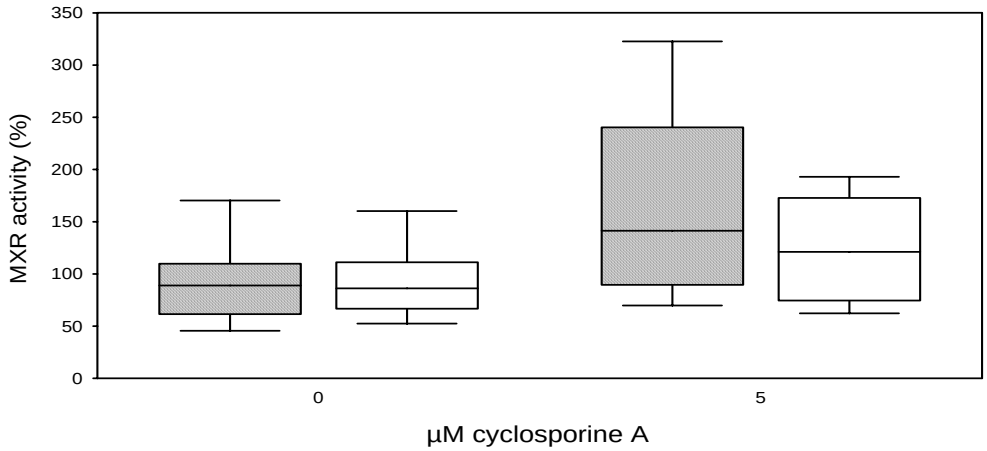

Figure 2. MXR activity in hemocytes from *M. edulis* after exposure to cyclosporine A. Shaded - female hemocytes; open - male hemocytes. The boxes are relative to median control. Lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

TBT effects

The viability in hemocytes seemed to decrease after exposure to TBT (figure 3.a and 4a.). At exposure to 3.16 nM TBT the metabolic activity in hemocytes sampled from female mussels decreased with 50 % (figure 3.a) (p=0.0001, Kruskal-Wallis), and at 31.6 nM and 316 nM TBT the metabolic activity had decreased with 90% compared with the metabolic activity in control group (significant with p=0.0001, Kruskal-Wallis). Membrane integrity decreased with 60% in female hemocytes compared to the control after exposure to 3.16 nM TBT (p=0.0001, Kruskal-Wallis) and there were no differences in respond between the three concentrations. MXR activity did not change from control at any of the tested concentrations in the female hemocytes (figure 3b).

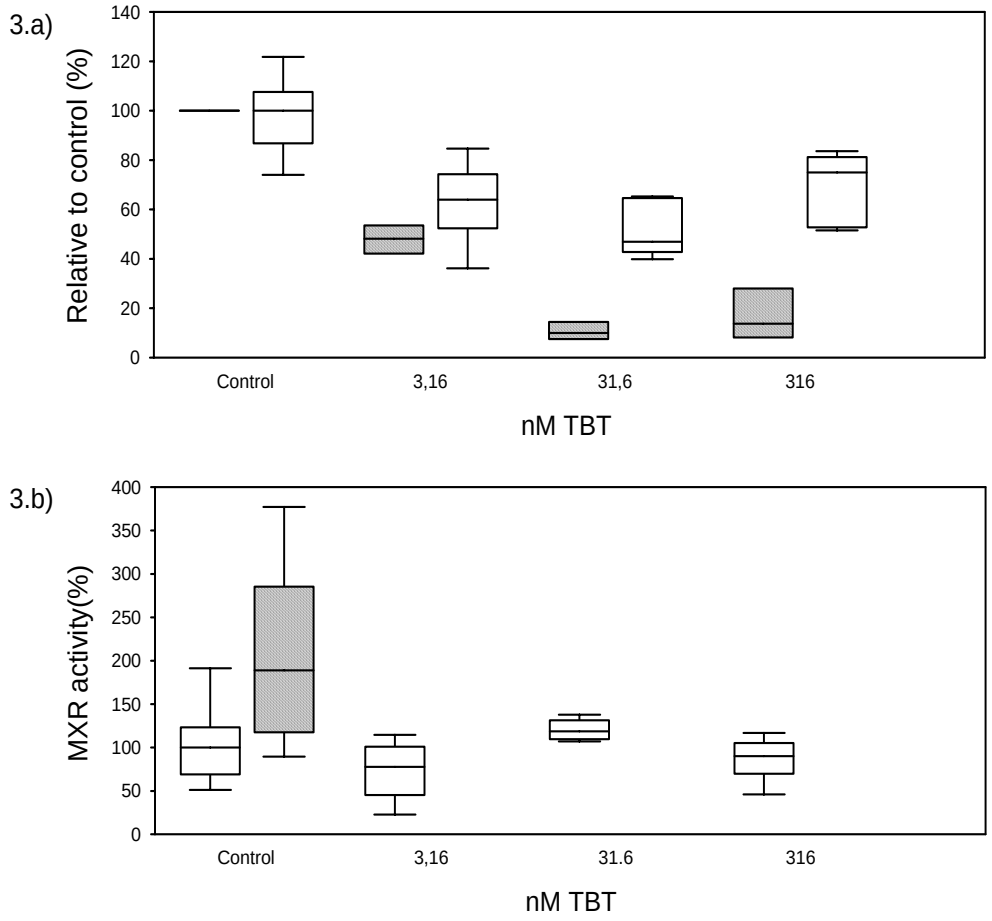

Figure 3. Effect of TBT on female hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM(membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

In hemocytes from male mussels (figure4.a) there was a significant decrease in metabolic activity after exposure to TBT (p=0.0001, Kruskal-Wallis). At 3.16 nM TBT the activity was 40% of control, and at 31.6 nM TBT 10%. At 31.6 and 316 nM TBT there was no differences in metabolic activity. Membrane integrity decreased with 50% compared to the control at 3.16 nM (p=0.0001, Kruskal-Wallis). There was no significant difference between 3.16, 31.6 and 316 nM TBT. In the MXR assay at 316 nM TBT the median rhodamine B accumulation was 195% compared to control (p=0.0088, Kruskal-Wallis). The two lower concentrations did not differ from control. Male hemocytes seemed to be more sensitive to TBT exposure in regard to cell viability and degree of MXR inhibition than female hemocytes.

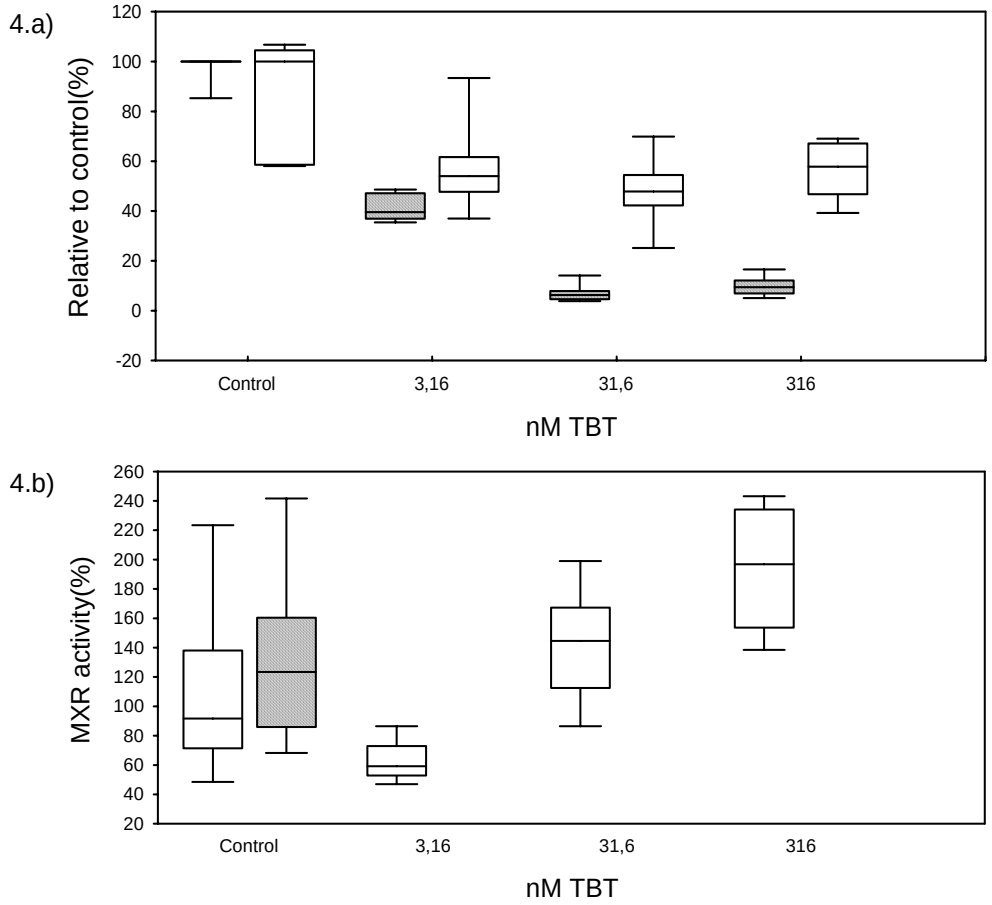

Figure 4. Effect of TBT on male hemocytes a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

HBCD effects

The viability in female hemocytes decreased after exposure to HBCD (figure 5a). After exposure to 5, 10 and 20 µM HBCD metabolic activity was significant different from control (p=0.001, Kruskal-Wallis). At 5 µM HBCD the activity decreased with 10% compared to control. There was no significant difference between the tested concentrations of HBCD. After exposure to 5, 10 and 20 µM HBCD membrane integrity in hemocytes sampled from female mussels decreased to 80% compared with control(significant at p=0.0002, Kruskal-Wallis), and there were no differences between the hemocytes treated with 5, 10 or 20 µM HBCD. In the MXR assay there was neither inhibition nor induction of MXR (Fig. 5b)

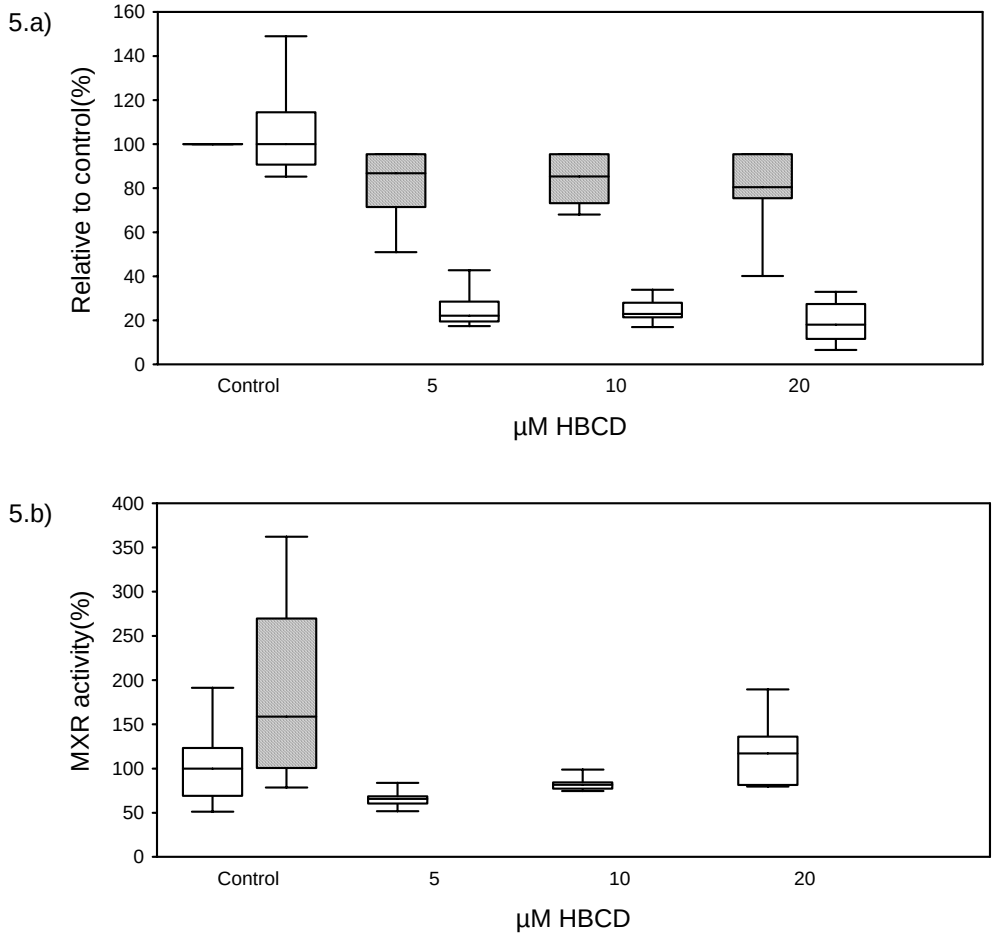

Figure 5. Effect of HBCD on female hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

The viability of male hemocytes decreased after exposure to HBCD (figure 6. a). Hemocytes treated with 5 µM HBCD had a median at 74% of the metabolic activity compared to control (significant with p= 0.0001, Kruskal-Wallis). Membrane integrity was 15% compared to control and significant with p=0.0002 (Kruskal-Wallis). Between the different concentrations there was no significant difference in effect on metabolic activity or membrane integrity. There was no difference in MXR activity in the hemocytes after exposure to HBCD. The male hemocytes seemed to be more sensitive than female hemocytes to HBCD exposure with regard to cell viability.

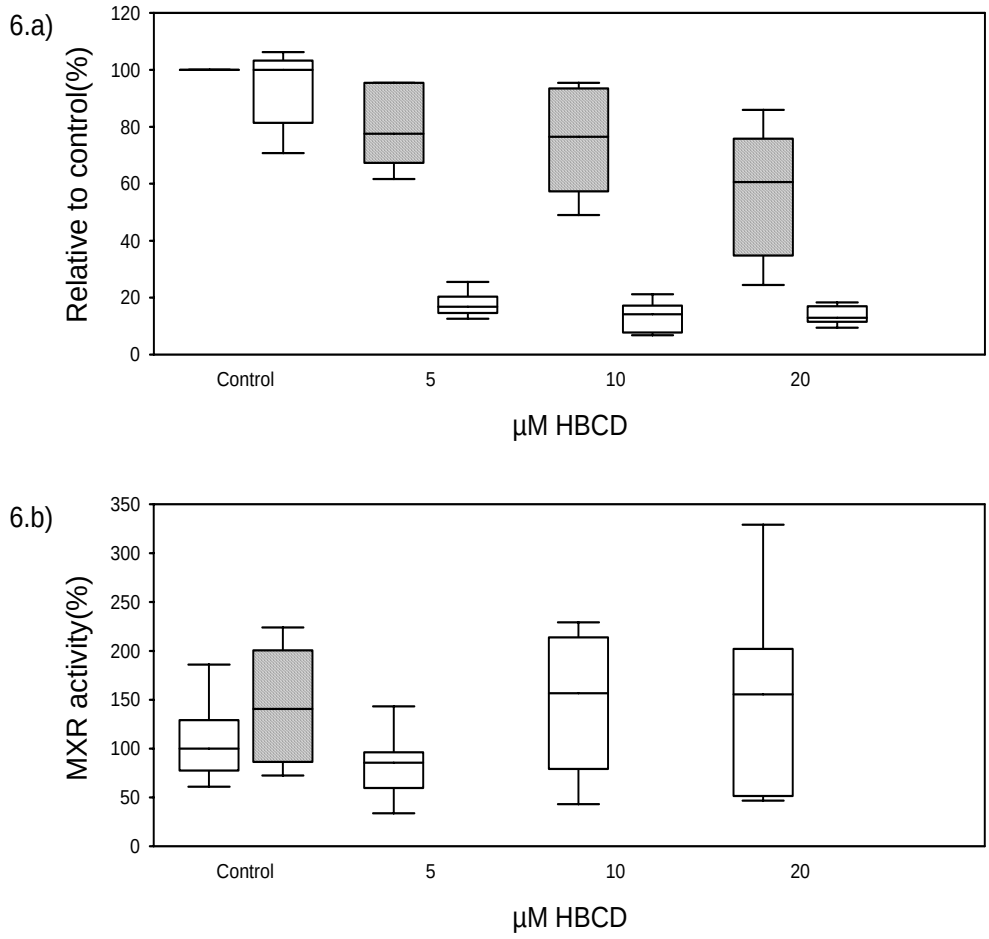

Figure 6. Effect of HBCD on male hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM(membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A;open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

TBBPA effects

Viability in female hemocytes decreased after exposure to TBBPA (figure 7a). Metabolic activity did not change after exposure to any of the concentration compared with control. Membrane integrity decreased after exposure to 5 µM TBBPA with 60% (significant with p=0.009, Kruskal-Wallis).There was no difference in respond between the different concentrations. The MXR activity did not change after exposure to the tested concentration of TBBPA (figure 7.b).

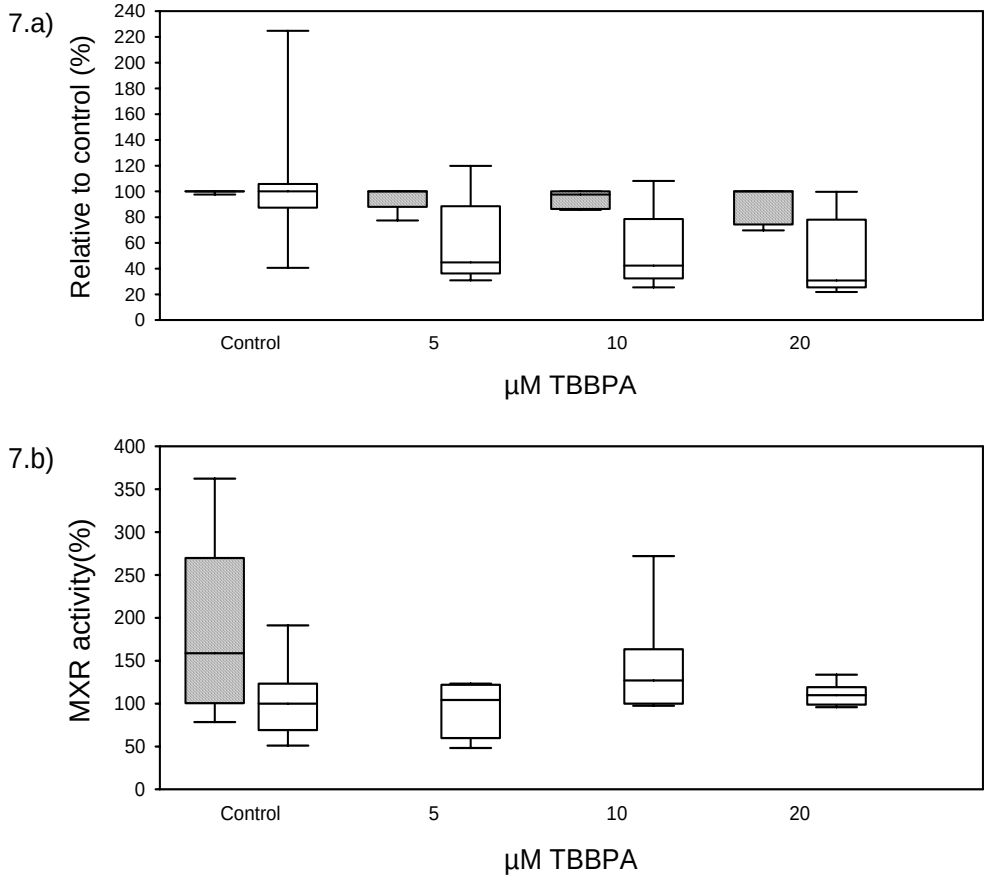

Figure 7. Effect of TBBPA on female hemocytes a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

Male hemocytes had a decrease in viability after exposure to TBBPA (figure 8.a). At 20 µM TBBPA the metabolic activity had decreased with 20% (significant with p=0.0013, Kruskal-Wallis) compared with control. At 5 µM there was no difference compared with control, but at 10 and 20 µM TBBPA the integrity have decreased with 30 and 50%. There was no change in MXR activity after exposure to TBBPA (figure 8.b). Male hemocytes seemed to be more sensitive to TBBPA exposure than female hemocytes with regard to cell viability.

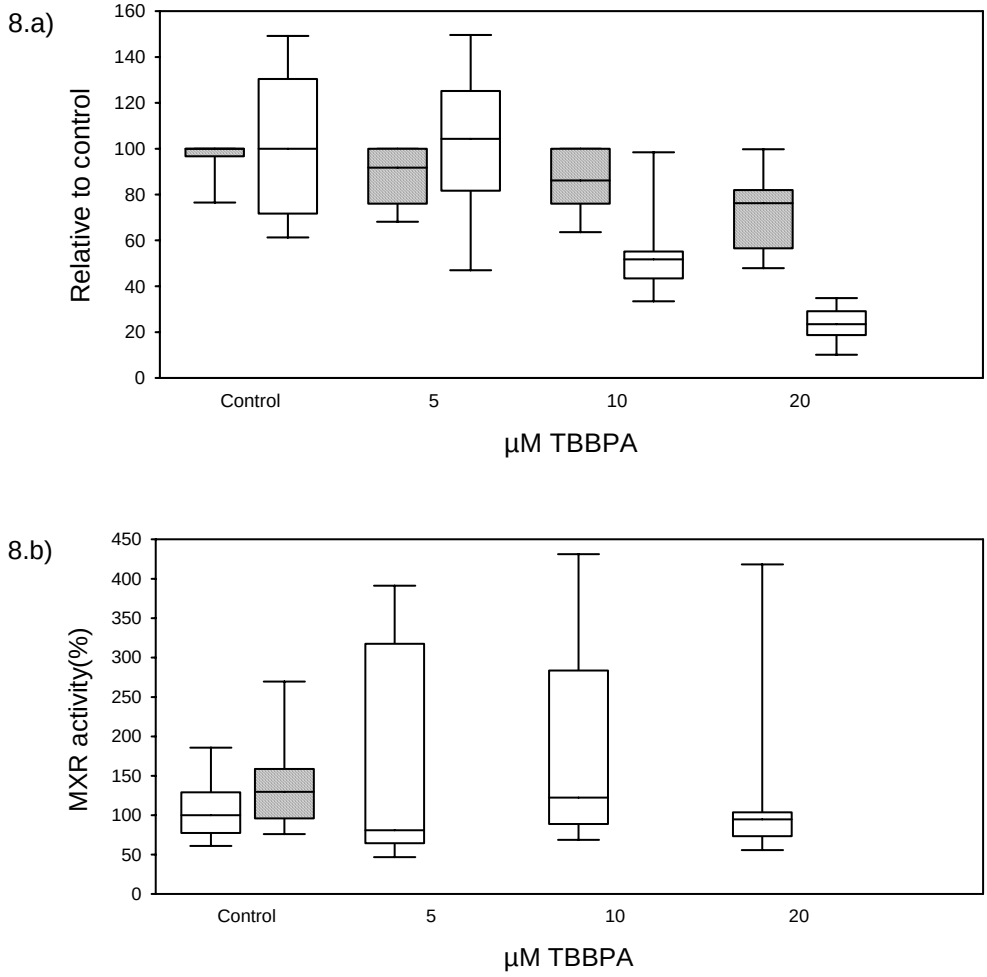

Figure 8. Effect of TBBPA on male hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

PFOS effects

The viability in female hemocytes did not change after exposure to PFOS at any concentration (figure 9.a.). MXR activity was strongly inhibited (figure 9.b). At 8 nM PFOS with an accumulation of 470% of the substrate compared to control (Significant with p=0.0001, Kruskal-Wallis). At exposure to 0.08 and 0.8 nM PFOS there was no significant effect on the MXR activity in female hemocytes.

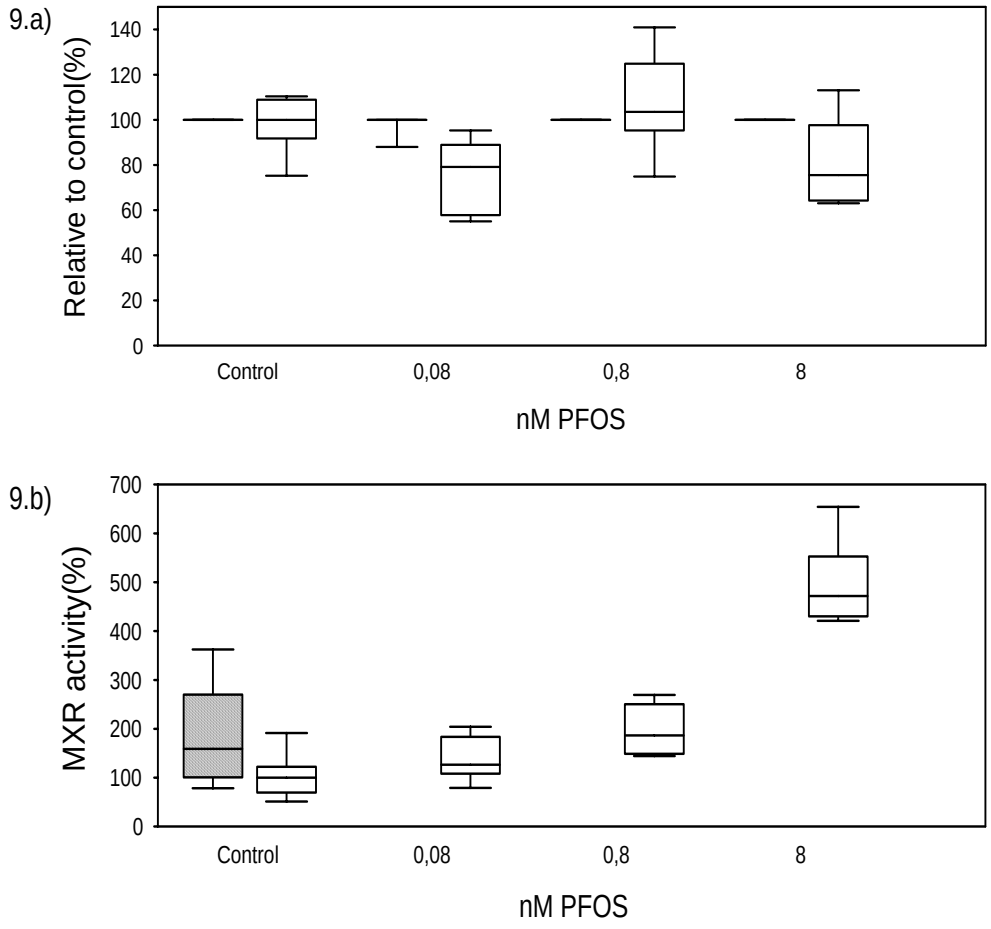

Figure 9. Effect of PFOS on female hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

Male hemocytes viability was not affected by PFOS at the tested concentrations (figure 10.a.). MXR activity in male hemocytes was not significant affected at 0.08 or 0.8 nM PFOS (figure 10.b.), but had a significant increase at 270% compared to control ($p=0.007$, Kruskal-Wallis) at 8 nM. The female hemocytes seemed to have a higher degree of MXR inhibition than male hemocytes.

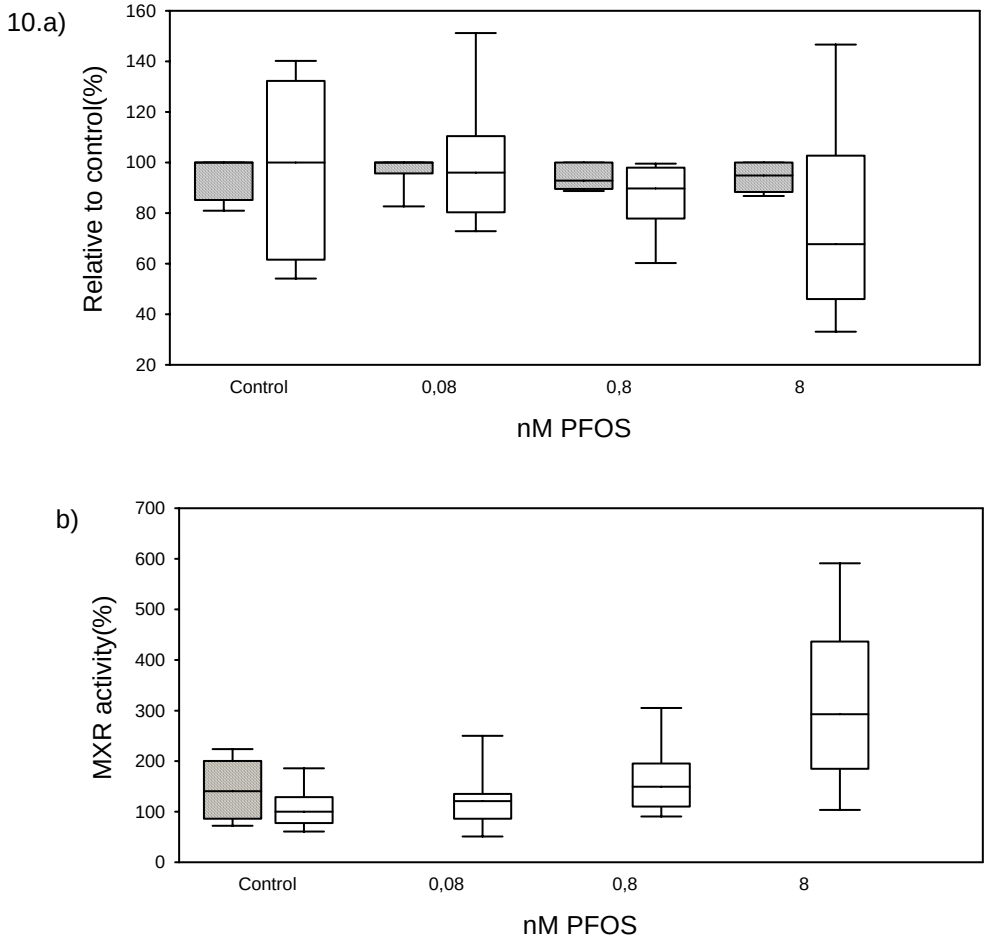

Figure 10 Effect of PFOS on male hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

PFNA effects

The exposure to PFNA did not affect viability in female or male hemocytes (figure 11.a. and 12. a.). MXR activity in female and male hemocyte exposed to 2 pM PFNA increased the accumulation (figure 11.b. and 12.b.). At 2 pM PFNA the female hemocytes had accumulated 200% compared to control (p=0.0057, Kruskal-Wallis).

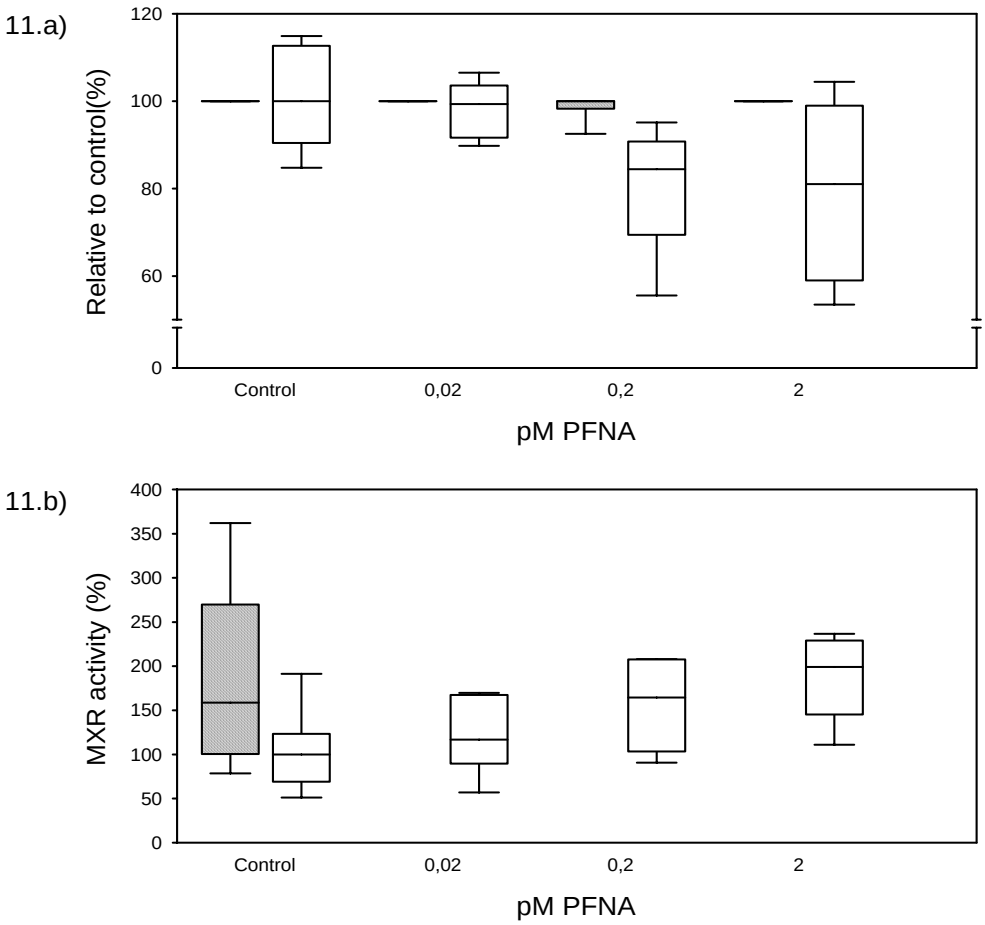

Figure 11. Effect of PFNA on female hemocytes. a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

Male hemocytes had accumulated approximately 170% compared to control (p=0.0001, Kruskal-Wallis). Female hemocytes showed a higher degree of MXR inhibition than male hemocytes.

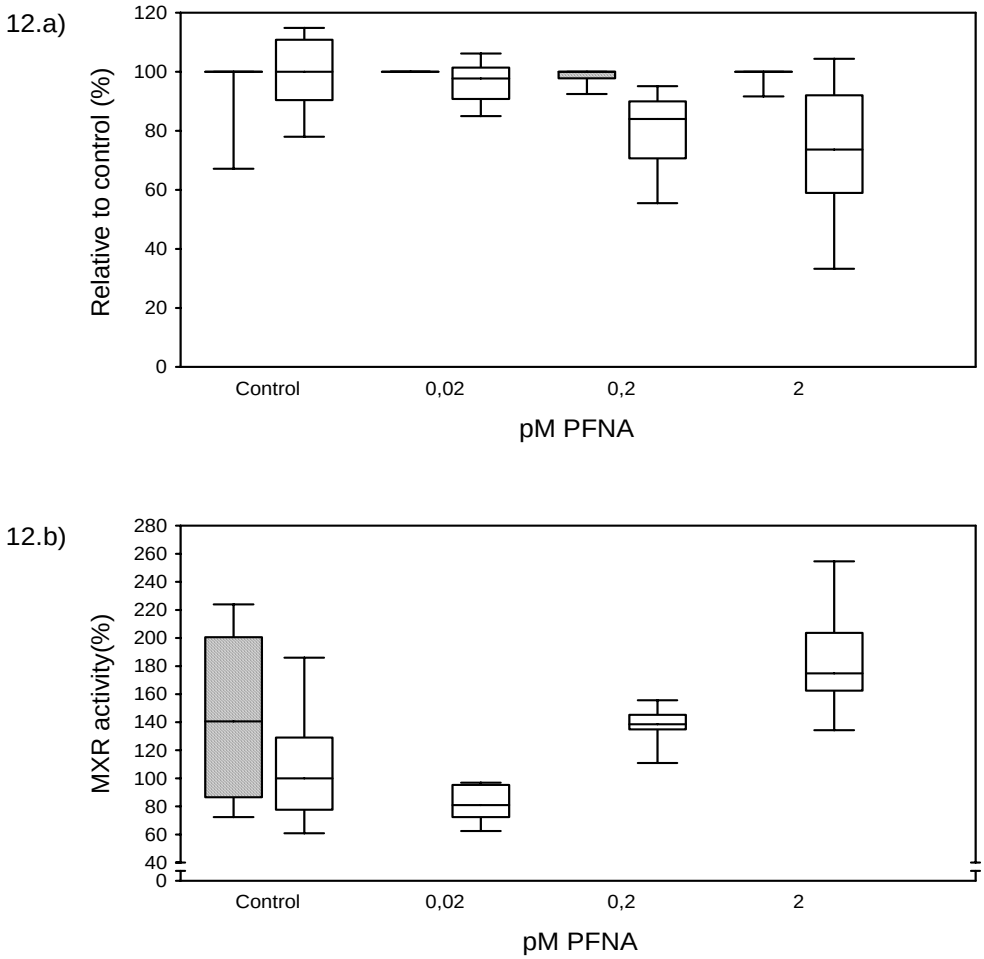

Figure 12. Effect of PFNA on male hemocytes a) Cell viability, shaded-alamar Blue (metabolic activity); open-CFDA-AM (membrane integrity). b) MXR activity, shaded boxes-inhibited with 5 µM cyclosporine A; open without cyclosporine A. Measurements with eight replicates; lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles.

Phenol equivalent

With the different contaminants and concentration the brominated flame retardants showed highest toxicity towards metabolic activity (table 2). PFNA and PFOS did not show any significant effect on metabolic activity. With the exposure to TBT there a clear effect in male hemocytes while female hemocytes were not affected to the same extent.

Table 2. Phenol equivalents calculated from the average metabolic viability. At female phenol equivalents 1mM is added due to the no effect level at the standard curve.

	HBCD		TBBPA		PFNA		PFOS		TBT	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Low	**2,7**	**2,7**	**1**	**1,34**	**1**	**0**	**1,5**	**1**	**1,7**	**4,3**
Median	**2,9**	**3**	**1**	**1,6**	**1,5**	**0**	**1,4**	**0**	**1,7**	**4,5**
High	**3,7**	**4,7**	**1**	**3,6**	**1**	**1,3**	**1,3**	**0**	**2,25**	**5,8**

Discussion

In this study hemocytes sampled from *M. edulis* were affected at different extent after exposure to the five contaminants. The perfluorinated compounds seemed to affect MXR activity while the brominated flame retardants and TBT where affecting cell viability. Hemocytes sampled from male mussels seemed to be more sensitive towards the contaminants with regard to cell viability and MXR activity showing a higher degree of decrease in activity.

Hemocytes from male and female *M. edulis* exposed to 3.16, 31.6 and 316 nM TBT seemed to have a decreased metabolic activity and membrane integrity. The most obvious effect was observed in metabolic activity. Studies conducted on the toxicity of TBT have shown several effects on cellular and individual level in aquatic organisms. After treatment with 50 µg TBT /l (158 nM) there was a 100 % mortality in the oysters *Crassostrea gigas* and rock shells *Thai clavigera* (Meng et al., 2005). Pathological studies have shown swelling and disrupting in mitochondria membrane cells in marine bivalves after exposure to TBT (Huang & Wang, 1995). The swelling increased the membrane 3-4 times compared to normal mitochondria membrane. They suggested that part of TBT`s toxicity could be caused by this effect on mitochondria. High doses of TBT has been shown to depress phagocytes in both fish blood cells and oysters hemocytes (Anderson, 1994) and the clam *Tapes philippinarum* (Cima et al., 1998). TBT has also been shown to enhance apoptosis in gill filament in mussels (Micic et al., 2001). A study to detect genotoxic damage to adult *M. edulis* decreased hemocytes viability after exposure to 0.5 and 1 µg TBT/L (1.58 and 3.16 nM) after 60 days (Hagger et al., 2005). With a decreased metabolic activity it might be difficult for the cell to maintain its normal activity, with the consequence of a depressed phagocyte activity or swelling of membrane. The decreased metabolic activity and membrane integrity in this study supports

other studies which imply a toxic effect on mitochondria and/or metabolism after exposure to TBT. Several studies conducted have shown that exposure in some marine snail species might lead to imposex, the development of a penis in marine snails (Axiak et al., 2003; Fernandez et al., 2005; Oberdörster & McClellan-Green, 2002; Pellizzato et al., 2004; Santos et al., 2005). The underlying mechanism has been suggested to be inhibition of aromatase activity leading to increased testosterone and decreased estradiol levels (Oberdörster & McClellan-Green, 2002) in female snails. There was a difference among male and female mussels with the male showing a higher relative decrease in metabolic activity after exposure indicating TBT being more toxic to these. The result in this study with male hemocytes being more sensitive might be caused by interspecies differences among different species of mollusks. Other studies conducted on the toxicity of TBT have shown interspecies differences between and within different phylum (Al-Ghais et al., 2000; Brown et al., 2004; Fournier et al., 2002). Another possibility is a decrease in immune defense as a respond to TBT exposure, followed by an increase in mortality among male individual. A laboratory study conducted on imposex in *Thais clavigera* showed a decrease in female rate compared with male animals (Shim et al., 2000) with increasing concentration of TBT rejecting this possibility of male mollusks dying to let the effect being undetected. MXR activity was inhibited at the highest concentration of TBT in male hemocytes. As there might be a decrease in membrane integrity at the lower concentration of TBT, the substrate content should be expected lower. This was not the case within the lower concentrations or within the exposed female hemocytes in this study. An inhibition of MXR activity and enhanced levels of substrate in this study might have been undetected as the membrane integrity was lowered, and not taken into consideration when the accumulation of rhodamine B was evaluated. The possibility of substrate leaching out might lead to a false assumption of a non-inhibited Pgp-pump as there are no difference between hemocytes without inhibitor and the hemocytes exposed to contaminants. Some adaptations have to be done to reveal such a possibility.

In a study conducted on the toxicity of HBCD in aquatic organisms there were found no acute toxicity. The same study conducted on mammals gave an effect towards thyroid hormone an increased liver weight at high concentration (Darnerud, 2003). In this study, membrane integrity was fare more affected than

metabolic activity in both male and female mussels. The hemocytes were exposed to a higher concentration in correlation with the findings of higher levels in mussels in nature than the other contaminants (Schlabach et al., 2002). A possible apoptosis in the hemocytes after high doses of HBCD might explain these results. During apoptosis the metabolic activity still remains as this type of cell death are controlled to prevent the other neighbouring cells from inflammatory responses while undergoing a cell death. Therefore, evaluating cell viability, metabolic activity might not be a good measure on cell viability alone. Metabolic activity had a lower activity after exposure in hemocytes from males than from females indicating a higher sensitivity in male mussels. There was no change in MXR activity, but with lower membrane integrity, the relative concentration of substrate could be higher in each cell as already mentioned.

Metabolic activity in female hemocytes was not affected by the tested concentration of TBBPA. A possible explanation could be the hemocytes undergoing apoptosis as in hemocytes exposed to HBCD with the result of being unchanged at al concentration. Membrane integrity decreased at 5 μM, but there was no difference between the different concentrations. Lower concentration than 5 μM might be cytotoxic to female hemocytes, and further testing at lower concentration has to be done to reveal this possibility. The accumulated substrate was not different from control, but as with TBT and HBCD the lowered membrane integrity might be influencing the outcome of the assay. Male hemocytes metabolic activity were more sensitive to TBBPA than female with the activity significantly affected at 5 μM TBBPA. The membrane integrity was less affected in male mussels. There was a slightly decrease in male substrate accumulation which might be a consequence of a possible undetected MXR induction as already discussed. Lowered concentration than tested could be tried to test if TBBPA might lead to an induction. TBBPA has been suggested to induce oxidative stress *in vivo* in rainbow trout (D. Ronisz et al., 2004), and in a recent study conducted on TBBPA and effect on hemocytes from *Mytilus galloprovincialis* a linear negative correlation on lysosomal stability and TBBPA concentration were found (Canesi et al., 2005). In correlation with these studies TBBPA most possible affect membrane integrity.

The tested concentration of PFOS did not affect viability in hemocytes from male or female mussels at tested concentrations. MXR activity was significantly affected with inhibition at all concentrations in both sexes. A field study conducted on freshwater fish transplanted into areas with known contamination of PFOS showed a damage of liver and gills after exposure. The same study also suggested a possibility that other contaminant present in the area could be the reason (Hoff et al., 2005). With the MXR inhibitory properties of PFOS found in this study the other contaminant present in the water might accumulate at a higher degree in the animals than without PFOS. Female hemocytes had a higher degree of inhibition than male hemocytes suggesting a higher degree of MXR activity or sensitivity. PFNA did not decrease the cell viability in hemocytes from male and female mussels. The agent inhibited MXR activity in hemocytes with a higher inhibitory activity on female hemocytes than male hemocytes.

In this study several different effect were found in the different groups of contaminant. The organic metal, TBT, had highest toxicity towards metabolic activity. Brominated flame retardants affected membrane in a higher degree than TBT. The PFOS and PFNA did not affect viability but highly enhanced the accumulation of substrate with MXR inhibition. As chemosensitisers this might enhance accumulation of toxins and lead to other injurious effects with the presence of other toxins or pollutant. Even though the concentration of TBBPA and HBCD were higher than the other contaminants, they showed a higher effect on membrane integrity but not on metabolic activity indicating higher membrane toxicity. As HBCD is a stronger lipophilic agent it might accumulate in membrane in a higher degree than mitochondria compared to TBT (SFT, 2004). TBT has been shown to posses a lower log K_{OW} than brominated flame retardants, and with the possible respond to affect different targets within a cell. As the membrane mainly consists of a lipid bilayer, the contaminants with highest log K_{OW} not surprisingly decrease membrane rather than metabolic activity. Female hemocytes showed a higher degree of MXR inhibition after treatment with PFOS, PFNA and cyclosporine A than male hemocytes, indicating a higher MXR activity. Seen in relationship with other studies, a higher MXR activity in female *M. edulis* might be suggested(Shim et al., 2000). In general female mussel hemocytes were less sensitive towards the pollutants tested. In accumulation and elimination test with oysters there found to be

a higher degree of elimination of TBT in female shells (Meng et al., 2005). A higher MXR activity might be the reason for the lower toxic effect observed in this study as this mechanism could protect the hemocytes from accumulation of xenobiotic. Viability assay as measure on cytotoxicity in hemocytes detect effects from low concentrations, revealed different effects from different chemical groups and within different sexes. The metabolic activity and membrane integrity did not correlate, but as different chemicals posses' different toxic mechanism, correlation might not be essential. MXR activity determination in hemocytes needs further improvement. As the MXR activity was only affected at the contaminants with lowest concentration the MXR inhibitory property giving the possibility of the same contaminant working in different ways at different concentrations. Mortality among hemocytes after exposure to pollutant was not taken into consideration in this study with the possibility of MXR inhibition being undetected. To detect MXR inhibitory activity, first the cytotoxicity should be determined at the desired concentration of pollutant tested. Second, accumulation of substrate should be compared with control containing this concentration. By using lower concentration without an effect on cell viability the contaminants in this study might show MXR inhibitory properties.

By using hemocytes early effects could be noticed by levels found in mussels, and then reversed. Effects were seen hemocytes at lower concentration than used in other experiments, and within contaminants where effects has not been yet demonstrated. Cell viability and MXR activity are easily conducted as parameters in cytotoxicity testing at several groups of chemicals with significant responses. As hemocytes react, and are easy to maintain in a culture they could be used with success *in vitro* toxicity testing.

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Del 3. Feltstudie

Cytotoxicity and MXR in hemocytes from mussels and coelomocytes from echinoderms; a field study in Kastela Bay, Croatia

Cytotoxicity and MXR in hemocytes from mussels and coelomocytes from echinoderms; a field study in Kastela Bay, Croatia

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Abstract

Within biomonitoring the common practice is to analyze the concentration of pollutants in the organisms. However, focus is shifting towards an approach which combines such chemical analysis with measurements of effects of the contaminants in the organisms. In this study, the objective has been to develop methods for such monitoring of effects in cells from two aquatic organisms, the blue mussel Mytilus edulis *and the sea cucumber* Holothuria tubulosa*. Both species are sedentary, common and widely distributed, but differ in habitat and feeding habits. The blue mussel inhabitates rocky bottoms and is a filter-feeder, while the sea cucumber often is found in muddy/sandy areas and is a deposit-feeder. The blue mussel is commonly used in environmental monitoring. The sea cucumber has not previously been used in a monitoring perspective. Hemocytes and coelomocytes from these species were used as biomarkers on pollution. Cell viability and MXR-activity bioassays were adapted and evaluated for use in this cell model in a field study in Kastela Bay, Croatia. The cell viability was assessed with the two parameters metabolic activity (measured as alamar Blue-reduction capacity) and membrane integrity (measured as CFDA-AM-reduction capacity). MXR-activity is measured as capacity to extrude a fluorescent model substrate from the cell. Pollution load has previously been shown to change MXR-activity and decrease cell viability in many aquatic organisms, although the assays have not yet been incorporated in environmental monitoring practice. The study area is known to be one of the most polluted areas along the eastern Adriatic coast. Organisms in this area is expected to experience severe environmental stress from a range of different pollution categories, such as industrial waste, pesticides, heavy metals and untreated sewage. Both assays gave significant results, but the MXR-activity assay appeared to be*

more sensitive. However, the importance of measuring several parameters simultaneously is emphasised, as this increases the understanding of the model system as a whole.

Introduction

Biomonitoring is a collective term for techniques that use living organisms to produce information about biotic and abiotic components of an environment. The basis for environmental monitoring today is the determination or prediction of contaminant levels in the environment, in animals and in their organs, and a comparison of these levels with threshold values and their known effects. This will provide information about which pollutants are present in the environment/indicator species, at which concentrations and where it can be found. But the presence of pollutants in an aquatic ecosystem does not by itself indicate deleterious effects. Measurement of any biological responses to an environmental chemical at individual level or below might however provide such information (van der Oost et al., 2003). A response to a chemical at this level has been defined as a biomarker by Walker et al. (2001). Although the notion that such chemical approaches should be combined with an effect-based biomarker approach, has been promoted for more than 25 years (Alabaster & Lloyd, 1980; Sarakinos et al., 2000), such an approach has still not been fully implemented.

As the interest in an effect-based approach has increased, so have efforts to develop new and reliable biomarkers. There are certain criteria which are important when choosing a biomarker. First, the assay to quantify the biomarker should be robust, relatively cheap and easy to perform and the biomarker response should be sensitive to pollutant exposure and effects in order to serve as an early warning parameter. Baseline data of biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant induced stress. The impacts of confounding factors to the biomarker response, underlying mechanism of the relationship between biomarker response and pollutant exposure and toxicological significance of the biomarker and organism should be well established (van der Oost et al., 2003). It has also been suggested that the biomarker should be non-invasive or non-destructive to allow or facilitate biological monitoring in protected or endangered species (Fossi et al., 1997). Using a non-invasive sampling technique

will allow for the same individuals to be sampled repeatedly and decrease the number of animals required in the assay and allow the monitoring to continue over a period of time.
When biomarkers are used in risk assessment, ideally the same biomarker should be measured in different species to obtain insight in the variation in sensitivity (den Besten, 1998). Therefore, the implementation of biomarkers in environmental monitoring requires the selection of vertebrate and invertabrate species which can be regarded as representative of the diversity in life strategies that are found in nature (den Besten, 1998). In addition to this, the species should be relatively stationary, robust against handling and culture keeping and well characterized in terms of biology and physiology so that the pattern of exposure is known and sources of uncontrolled variation can be minimized.

Bivalves are dominant members of coastal and estuarine communities and have a widespread geographical distribution. They are sedentary and relative tolerant to a wide range of environmental conditions. The population are large and can bear repeated sampling, and they can easily be transplanted into areas of interest. Mussels are also filter feeders that pump large volumes of water and concentrate pollutant in their tissues and make it available to other trophic level in the aquatic community. They play an important part of the food supply in the predator community (Widdows & Donkin, 1992).

Holothuria tubulosa inhabitates muddy, corse and fine sands and detritic bottoms, and dominates the soft-bottom community in shallow areas in the Adriatic (Zavodnik, 1971). *H. tubulosa* is a deposit feeder and by its occurence in coastal and estuarine waters it is directly exposed to anthropogenic contaminants. The contaminant exposure is expected to differ from the exposure in blue mussels: while the mussels will be exposed to water-fraction and DOM-fraction of contaminants, sea cucumbers will to a greater degree be exposed to the sediment fraction (note that this fraction also will contain DOM from the water column).

For the cell based assays in this study, hemocytes and coelomocytes were chosen as model cells. Hemocytes and coelomocytes represent the most important internal defense mechanism in marine bivalves (Gosling, 2003) and echinoderms (Smith &

Davidson, 1992), respectively. Hemocytes are not confined to the hemolymph system, but moves freely in and out of the sinus into surroundings connective tissues, mantle cavity and gut lumen. The hemolymph also plays an important role in gas exchange, osmoregulation, nutrient distribution and elimination of wastes. The cells react to foreign substances and by phagocytosis or encapsulation, and infection is generally accompanied by intense proliferation of hemocytes. It is suggested that the intensity of the proliferation could be used as a quantifiable determination of the immunodefence response to physiological or pathological stress. Various pollutants are known to exert adverse effect on the immunity, and this can affect resistance to infection and thus influencing survival (Gosling, 2003). Coelomocytes are circulating cells in the echinoderm body cavity. Sea urchin coelomocytes have been shown to respond to stress conditions (Matranga *et al.* 2000) and has also been used as indicators of pollution (Matranga *et al*. 2000). To our knowledge, coelomocytes from holothurians have not previously been used as models for contamination effects.

Assays of cell viability assays have a broad application both within cell culturing and in the use of *in vitro* toxicology. In this experiment, the two molecular probes alamar Blue™ and CFDA-AM were used in a cell viability assay. Alamar Blue™ is reduced by cellular reductases, possibly by diaphorases or by NADH dehydrogenase (O'Brien et al., 2000)to form a fluorescent product. The reduction has been believed to take place on the mitochondria of the cells, but no evidence for this was found by O'Brien, Wilson et al (2000). The probe is water soluble and can diffuse freely along the concentration gradient in both reduced and oxidized form. Herein, alamar Blue™ fluorescence will be referred to as metabolic activity of the cells. The esterase activity is measured by the molecular probe 5-carboxyfluorescein diacetate, acetoxymethyl ester, CFDA-AM. This probe is converted by the non-specific esterases in living cells from a non-polar, fluorescent dye. The substrate diffuses rapidly into the cell while the product diffuses slowly out of the cells. Metabolized CFDA-AM can be read fluorometrically. The fluorescence readings will reflect the membrane integrity of the cells. This because of either reduced esterase activity when the cytoplasmic environment is disturbed in cells with lower membrane stability, or because metabolised CFDA-AM diffuses more rapidly from cells with lower membrane integrity. Because the two probes are measured at different

emission wavelengths both dyes can be added together to perform the assay in one single step, and provide a measure for cell viability. This is will both reduce time and cost spent on the assay. The probes do not have to be removed from the wells, and the assay may be conducted immediately after the incubation without further procedures (Ganassin et al., 2000).

The ability of aquatic organism to live and reproduce in polluted areas, and hold cell and tissue levels of contaminants below observed concentration in the surrounding environment indicates a well working defence system. It has been shown that aquatic organisms possess a mechanism similar to the multidrug resistance phenomenon observed in tumour cells resistant to anti-cancer drug (Keppler & Ringwood, 2001; Kurelec, 1992; Kurelec & Pivcevic, 1991; Minier & Galgani, 1995). I Induction of this mechanism, named multixenobiotic resistance (MXR), has been reported in numerous studies of aquatic organism after exposure in laboratory or in environment by anthropogenic contaminants or natural stress (Eufemia & Epel, 2000; Kurelec et al., 1995; Minier et al., 2000; Minier & Moore, 1996; Smital et al., 2003). If the cells have a mechanism to remove harmful compounds from their environment it might prevent injurious effects. MXR is caused by energy-dependent pumping of substances out of the cells. This removal is conducted by certain transmembrane proteins, probably can several different proteins act at the same time in one cell and the substrates are both endogenous chemicals and xenobiotics. The accumulation and toxic effects of substances in the cells, can thus be prevented (Kurelec, 1992). The protective role of the defence appears to be fragile: As opposed to MXR induction, it is also demonstrated that there are many classes of chemicals which are capable of inhibiting the MXR function. These are referred to as chemosensitisers, and can be environmentally hazardous chemicals, because they may lead to accumulation of xenobiotics and elevate internal levels of toxins (Smital & Kurelec, 1998). Elevated MXR-activity might occur via multiple mechanisms, and the factors which might explain it are not fully understood (Bard, 2000).The induction or inhibition of MXR has not yet been established as a biomarker on environmental pollution (van der Oost et al., 2003).

The objectives of this study were to develop non-invasive biomonitoring techniques, using coelomocytes from echinoderms and hemocytes from bivalve mollusks as

models and to adapt cell-based bioassays with MXR-activity and cell viability as endpoints.

Materials and methods

Study area

The study took place in October 2004 in and around Kaštela Bay (Figure 1). Kaštela Bay is the largest bay in the central part of Croatian coast with a total surface area of 60 km2 and an average depth 23 meters Annual mean salinity is around 34 in the surface layer and 37 in bottom layers and the water renewal period of the entire bay is 1 month (Ujevic et al., 2000). The bay is known as one of the most polluted areas of the eastern Adriatic coast, largely as a consequence of fast industrialization and urbanization without development of appropriate urban infrastructure, in particular of a wastewater collection and disposal system (Margeta, 2001). Today approximately 32 million m^3 of untreated municipal waste water and 20 million m^3 of partially treated industrial waste waters are discharged into the bay annually (Ujevic et al., 2000). Major industries located along the coast are shipbuilding, cement production, chemical factories and metal processing. In the towns of Kaštela and Trogir only 25% of the inhabitants are served by municipal sewage treatment works. The bay is contaminated by heavy metals, particularly mercury (Margeta, 2001), but also by lead, cadmium and manganese (Ujevic et al., 2000). After chemical analysis of the tissues from *M. galloprovincialis* from the bay, high concentrations of heavy metals, PCBs and PAHs were found (Ruus, personal communication). While the water inside the bay is heavily polluted, the area around Šolta is viewed as having a relatively low pollution load and served as a control site for the study.

Collection of blue mussels and sea cucumbers

Mytilus galloprovincialis were collected at five different locations inside and outside the Kaštela Bay (figure 1). The shipbuilding yard in Trogir, Adriavinil, which is close to an old factory believed to be a source of mercury, and Vranjic, close to the main sewage discharge from Split. All three stations are located inside the bay. Mussels from these places would be exposed to a range of pollutants such as mercury and lead, antifouling containing organic tin compounds, pharmaceuticals and halogenated hydrocarbons. Outside the bay, cultured mussels from a shellfish

farm and from the island of Šolta were used. Ten mussels were sampled from each station. At the shipbuilding yard in Trogir only five were sampled because of high mortality among the caged animals. The study period was outside the spawning season for *M. galloprovincialis*. The sizes of the mussels were 4-8 cm. The mussels were kept in big tanks with aerated surface water from the Šolta sampling site overnight.

Sea cucumbers (*Holothuria tubulosa*) were collected by bottom trawling at two locations, one inside Kaštela Bay and one close to the island Šolta (Figure 1). The study period is outside the *H. tubulosa* spawning season (Despalatovic et al., 2004). The collection of animals at Šolta station was done between 40 and 50 m depth. In Kaštela Bay, collection was done at 30-40 meters. The animals were kept in tanks with aerated surface water from the respective sites until sampling of the coelomic fluid.

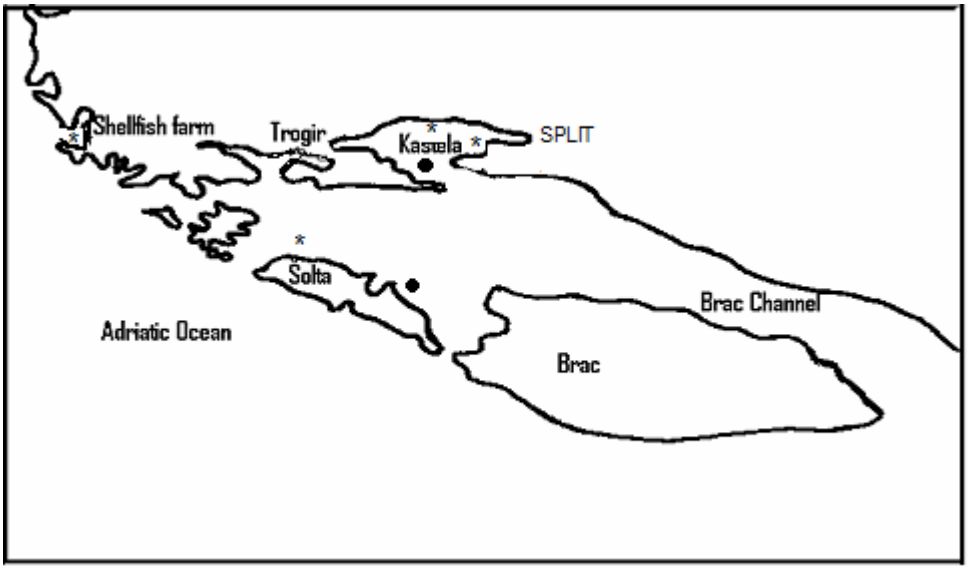

Figure 1: Kaštela Bay and surroundings, Croatia; sampling locations are indicated by circles (mussels) and stars (sea cucumbers).

Sample preparations

Sampling of hemolymph from blue mussels and coelomic fluid from sea cucumbers took place within 24 hours from collection of the animals. The sampling was done using a 1 ml syringe with a 23 gauge needle, both pretreated with cold PBS (36‰ w/v NaCl). Hemolymph from the blue mussels was withdrawn from the posterior adductor muscle and put in individual glass tubes containing 1 ml PBS (36‰ w/v NaCl) and the sample was further diluted with an equal amount of PBS (36‰ w/v

NaCl). From the sea cucumbers, 3-5 mL of coelomic fluid was withdrawn from each individual and the coelomic fluid diluted 1:4 in cold PBS in separate glass tubes. Cell suspension from each individual was seeded into the wells of 96 well microplates, with 200 µl pr well, in 8 replicates pr. individual. The cells in the microplates were then incubated in the dark at room temperature for 24 h to achieve a confluent monolayer at the bottom of each well.

Quantification of hemocytes

Each individual sample was fixed in paraformaldehyde (50:150 µl) and stored in 1.5 mL eppendorf tubes. Quantification of the hemocytes in this fixed cell suspension was done using a Multisizer^{TM} 3 Coulter counter with size settings ranging from 3-15 µm.

Quantification of coelomocytes

Coelomocytes were quantified using the commercial kit Quick Start^{TM} Bradford protein assay from Biorad using Bovine serume albumine standards (Bradford, 1976).

Cytotoxicity assay

Cell viability was assessed by the assay simultaneously using alamar Blue for metabolic integrity and CFDA-AM for membrane integrity. The protocol is adapted from (Ganassin et al., 2000): After 72 hours the media was carefully removed from the wells and 100 µl PBS (36‰ w/v NaCl) containing 4µM CFDA-AM and 5% (v/v) alamar Blue^{TM} was added to each well. The microplates were incubated for 30 minutes in room temperature in the dark. The fluorometric readings were performed on the plate reader Fluorolite 1000 Dynatech. Excitation and emission wavelengths were 485nm and 530nm respectively for CFDA-AM and 530 nm and 590 nm respectively for alamar Blue. The dyes are light sensitive, and every step of the protocol was carried out in the dark.

Results were expressed separately for the two probes, and on the mean of replicates for each individual. Different parameters for membrane integrity and metabolic integrity can be derived from the fluorometric readings. For blue mussels the parameters used are fluorescence values relative to the median of the fluorescence

values for cells from the control site Šolta. For sea cucumbers the parameters used are fluorescence relative to protein content of the individual samples.

Quality control of the cytotoxicity assay

An internal standard followed all microplates for the cytotoxicity assay, so that variation between different plates could be monitored.

Multixenobiotic resistance assay

In order to find the most suitable MXR reagents among the reagents available for the species in this study, as well as the best suited concentration of inhibitor, a preliminary test was conducted. The three dyes Rhodamine B, Rhodamine 123 and Calcein AM and the three MXR-inhibitors verapamil, cyclosporine A and MK571 were included in this test. Final concentrations were 0.1 µM for all three dyes and 0.1 µM, 1 µM and 5 µM, respectively, for all three MXR-inhibitors. The test was conducted on pooled hemolymph from the shellfish farm and pooled coelomic fluid from sampling site Šolta. All combinations of dye and inhibitor were tested in triplicates.

The preliminary tests lead to the decision to use different procedures for the MXR-assay for the two model species. For blue mussels preliminary testing resulted in the use of Rhodamine123 (0.1 µM) as a dye, and no MXR-inhibitor. The assay was performed on individual samples. 20 µl media from each well was removed and replaced with 20 µl Rhodamine 123 to obtain final wanted concentrations in the wells. After 1 hour incubation in the dark at room temperature, the cells were washed by removing 100 µl solution from each well and replace it with 100 µL PBS (36 ‰ w/v NaCl). This washing step was repeated once. After the second washing step, all solution was carefully removed from each well, and 100 µL Triton X-100 (0.1% v/v in PBS) was added. Fluorescence measurements were performed on the fluorescence plate reader, with excitation and emission wavelengths of 485 nm and 530 nm, respectively. MXR-activity was expressed relative to the reference site Šolta, which was done by dividing the median of fluorescence values for individuals by a random chosen value originating from the control site Šolta.

For sea cucumbers the assay on was performed on individual samples, using 0,1µM Rhodamine B and 0,1 µM Cyclosporin A. 100µlof the cell suspension in the well was removed and 50 µl of dye and 50 µl of the chosen inhibitor was added to achieve the wanted final concentrations in the designated wells. The rinsing and fluorometric readings were as described for blue mussels. Fluorescence measurements were adjusted for protein content in the respective sample. Some samples had to be discarded and the final number of individuals was 10 from Šolta and 5 from Kaštela. Each individual was analysed in four replicates, and analyses are performed on the mean of these replicates. MXR-activity is expressed as the ratio of fluorescence measurements when no model Pgp-inhibitor is present and measurements with the model Pgp-inhibitor Cyclosporin A present.

Quality control for the MXR-assay

An internal standard followed all microplates for the MXR-assays, so that variation between different plates could be monitored.

Statistical analysis

Statistical analyses were performed with Statistica 6.0 from StatSoft, Inc.

H_0: "There are no differences between cells originating from blue mussels sampled at the different stations". The data were analysed with Kruskal-Wallis analysis. Significant level for the rejection of H_0 was set to $p \leq 0.012$ according to Bonferroni correction with n equals 5 (Fisher & van Belle, 1993) .

H_0: There are no differences between cells originating from sea cucumbers collected near Šolta and in Kaštela. Level of significance for the rejection of H_0 was set to $p<0.05$. Normality of distribution for each sampling station was assessed in a normal-probability plot. Data that were found to be normally distributed were analysed using t-test. Data that were found not to be normally distributed were log-transformed and log-transformed data assessed for normality of distribution prior to t-test.

Results

Quantification of hemocytes

All results for hemocyte content and shell size were expressed relative to values from randomly chosen individuals collected at the reference site Šolta. The cultured mussels from the shellfish farm had a significantly higher concentration of hemocytes in their hemolymph compared to Šolta mussels (Kruskal-Wallis test, p=0.0009) and the highest median concentration of all stations (figure 3). These mussels also had a significantly larger shell size (figure 2) (Kruskal-Wallis test, p= 0.0006). The animals from Adriavinil were significantly smaller than the Šolta mussels (Kruskal-Wallis test, p=0.0013) Concentration of hemocytes in the hemolymph did not show any significant differences. Animals from Vranjic had a significantly higher concentration of hemocytes (Kruskal-Wallis test, p=0.0012). The size was not significantly different from the reference. From the shipbuilding yard in Trogir no significant differences in size or concentration compared to reference were found.

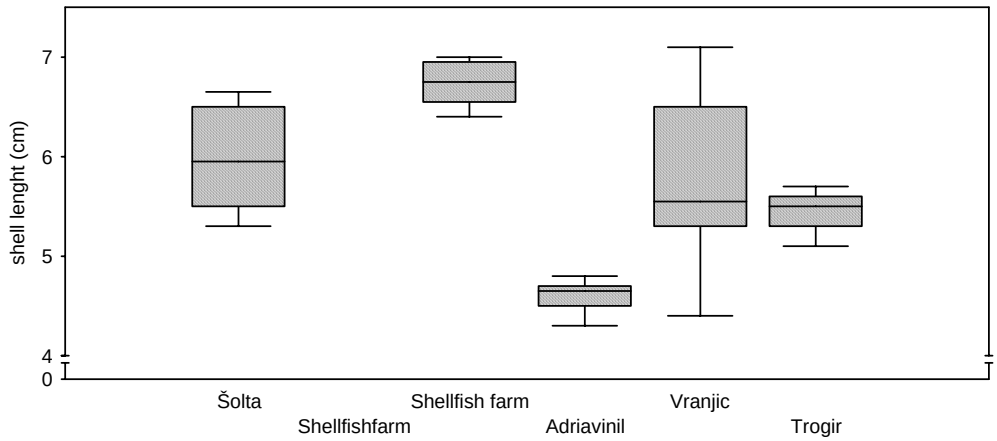

Figure 2, Length of the shell from mussels (*Mytilus edulis*) collected at five different sampling sites (n=10, ecxept from Trogir station: n=5). Lines are median length, boxes are quartiles and whiskers are 10 and 90 percentiles. Scale break from 0.5 to 4.0 cm on the Y-axis.

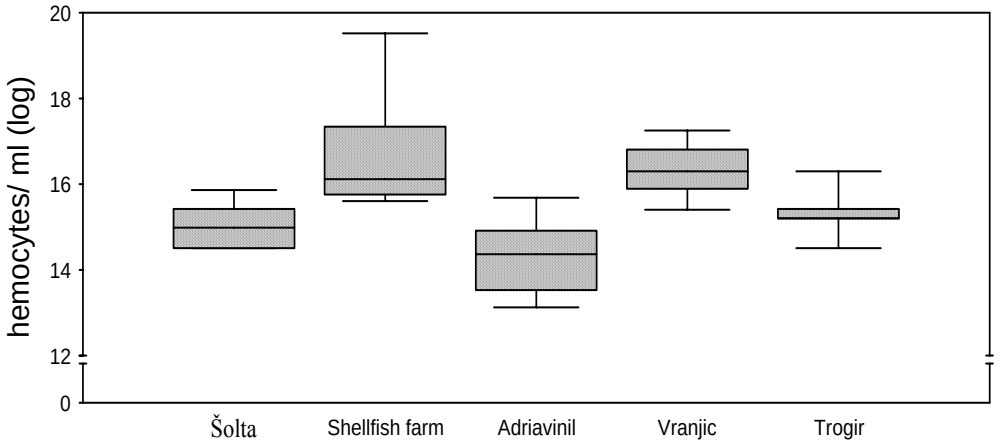

Figure 3, Concentration of hemocytes in the hemolymph in animals from five different sampling sites. Lines are median concentration, boxes are quartiles and whiskers are 10 and 90 percentiles. Scale break from 1 to 12 cm on the Y-axis.

No significant differences between stations in protein content in the samples were found in this experiment (figure 4).

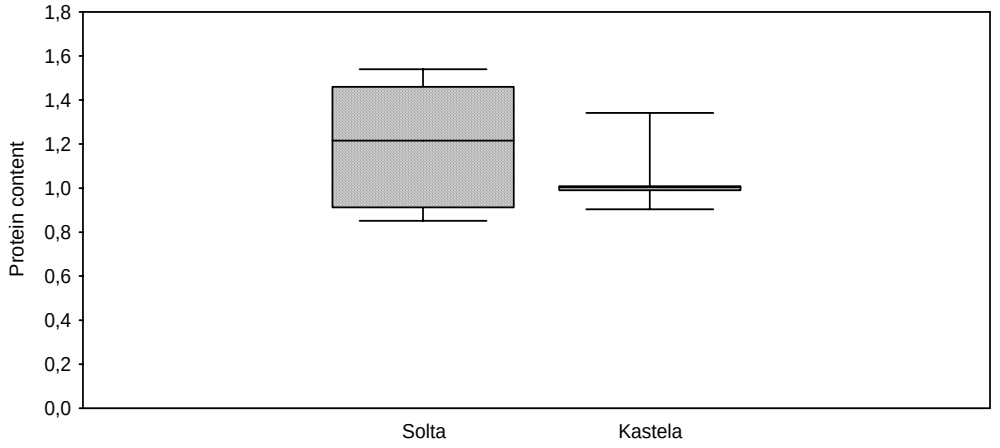

Figure 4: Protein content in sea cucumber (*Holothuria tubulosa*) coelomic fluid samples taken from individuals at sampling station Šolta (n=10) and sampling station Kaštela (n=5). Lines are median, boxes are quartiles and whiskers are 10 and 90 percentiles.

Cell viability assay

Results for the cell viability assay on mussels are expressed relative to the median values from the reference site Šolta. Hemocytes from the mussels collected at the island of Šolta (figure5) had the highest metabolic integrity, and hemocytes from animals collected at Adriavinil showed the lowest activity with 0,6 of the activity compared to Šolta (Significantly different, Kruskal-Wallis test, p=0.0002). From the shipbuilding yard in Trogir hemocytes had 0.7 metabolic integrity compared to Šolta (Kruskal-Wallis test, p=0.0002) At the site Vranjic the hemocytes had an activity at 0,8 (Significantly different, Kruskal-Wallis test, p=0.0001).There were

not found any significant differences in metabolism were found between hemocytes from cultured mussels from the shellfish farm and mussels from Šolta.

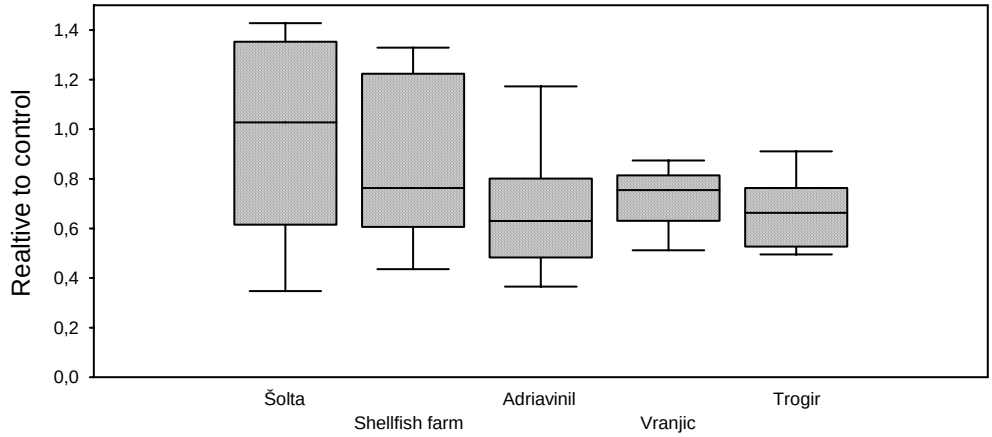

Figure 5: Metabolic integrity measured at five different sampling sites. Metabolic integrity in hemocytes was measured by the molecular probe alamar Blue. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Membrane integrity had a large variation from in the four sites Adriavinil, Šolta, Shellfish farm and Vranjic, but only in the hemocytes sampled from the caged mussels in Trogir had a significant lower integrity compared with integrity in mussels from Šolta (Kruskal-Wallis test, p=0.001) The membrane integrity being 0,2 of the values from Šolta (figure 6).

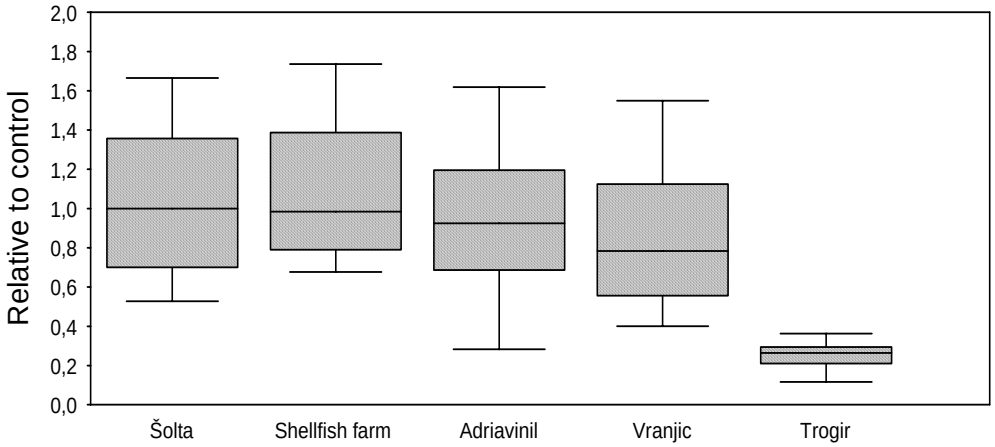

Figure 6: Membrane integrity measured at five different sampling sites. Membrane integrity in hemocytes was measured by the molecular probe CFDA-AM. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Metabolic integrity in sea cucumber coelomocytes have been expressed as alamar Blue^{TM} fluorescence units relative to protein content of the individual samples (figure 7). Metabolic integrity in sea cucumber coelomocytes were found to be

normally distributed in a normal-probability plot. Metabolic integrity was significantly higher in Kaštela cucumbers compared to Šolta cucumbers (t-test; p=0.000002).

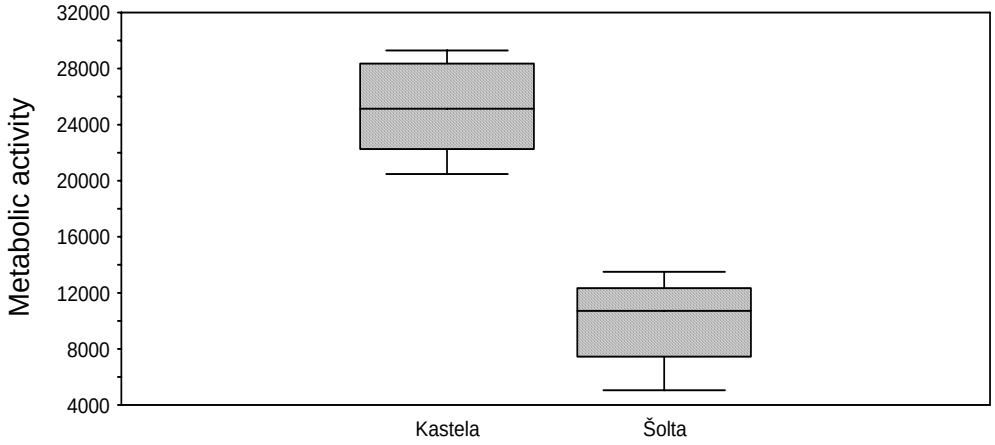

Figure 7: Metabolic integrity measured in sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Metabolic integrity in coelomocytes was measured by the molecular probe alamar Blue. Metabolic integrity given as fluorescence units pr. protein content in the sample. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Membrane integrity in sea cucumber coelomocytes is expressed as CFDA-AM fluorescence units relative to protein content of the individual samples (figure 8). Membrane integrity in sea cucumber coelomocytes were found to be normally distributed in a normal-probability plot. Membrane integrity was significantly higher in Kaštela cucumbers (t-test: p=0.000007).

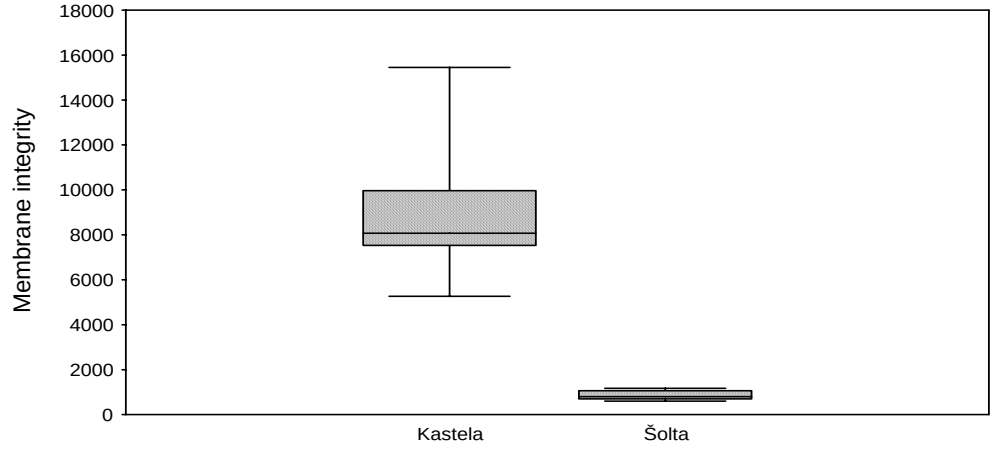

Figure 8: Membrane integrity measured in sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Membrane integrity in coelomocytes was measured by the molecular probe CFDA-AM. Membrane integrity given as fluorescence units pr. protein content in the sample. Lines are median, boxes are quartiles and whiskers are 10 and 90 percentiles.

Multixenobiotic Resistance Assay

Fluorescence of the accumulated substrate Rhodamine 123 in hemocytes is related to MXR activity with high fluorescence in cells with high concentration of substrate Rhodamine 123 and low MXR activity (figure 9). Mussels from the unexposed island Šolta had the highest accumulation of the substrate Rhodamine 123 in the hemocytes. The mussels from Trogir had accumulated an average about 0.6 of the possible substrate, and was significantly different from control cells (Kruskal-Wallis test, p= 0.001). The cultured shell from the shellfish farm had a MXR activity ratio at 0.4 compared to control (Kruskal-Wallis test, p= 0.001), the mussels from Adriavinil had 0.3 compared to control (Kruskal-Wallis test, p= 0.001) and from Vranjic 0.2 compared with control (Kruskal-Wallis test, p= 0.001). There were no differences in the ratio of substrate in the four sites Shellfish farm, Adriavinil, Vranjic or Trogir.

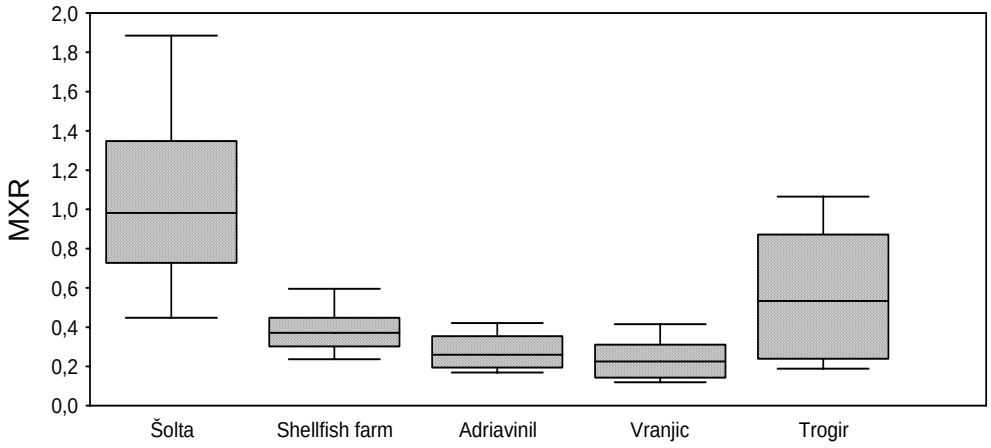

Figure 9: Accumulated Rhodamine 123 in mussels from five different sampling sites. Content of Rhodamine indicates degree of MXR activity with low content of Rhodamine 123 as a response to high MXR activity. The fluorescence from accumulated Rhodamin 123 was related to the median accumulated Rhodamine 123 in mussels from Šolta. Lines are medians, boxes are quartiles and whiskers are 10 and 90 percentiles.

Significant differences were found in median Rhodamine B fluorescence values in sea cucumber coelomocytes between different stations (figure 10). Both fluorescence measurements with inhibitor present and the ratio of the two fluorescence measurements showed significant differences between stations. Fluorescence with inhibitor was significantly higher at the Kaštela station compared to the Šolta station (t-test; p=0.028187). No significant differences were found in fluorescence without Pgp-inhibitor present.

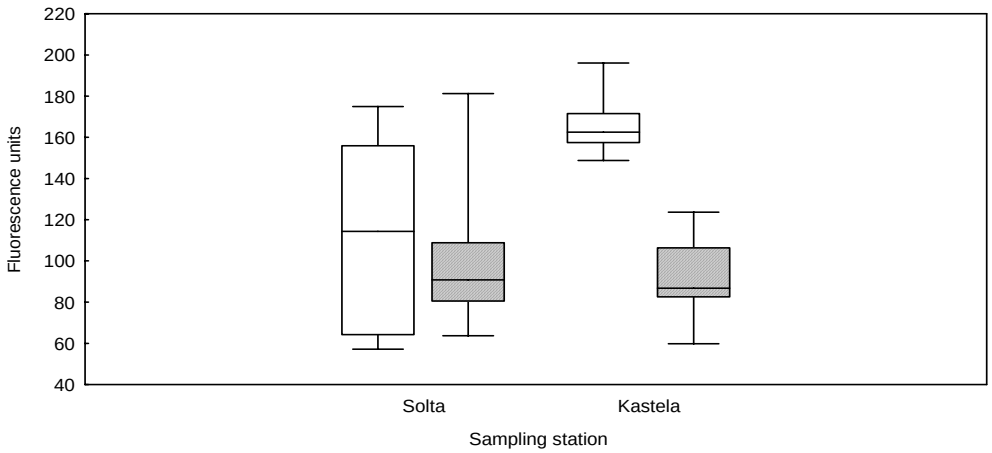

Figure 10: Rhodamine B fluorescence measurements on sea cucumber (*Holothuria tubulosa*) cells taken from individuals sampling station Šolta (n=10) and sampling station Kaštela (n=5). Fluorescence measurements with Pgp-inhibitor present (open boxes) and fluorescence measurements without Pgp-inhibitor present (shaded boxes). Lines are median fluorescence values, boxes are quartiles and whiskers are 10 and 90 percentiles.

The ratio of the two fluorescence measurements (figure 11) was significantly lower at the Kaštela station compared to the Šolta station (T-test; p=0.020010). The invertion of this ratio provides a measure of MXR-activity in the organisms at the stations, this activity thus being higher in the Kaštela station compared to the Šolta station.

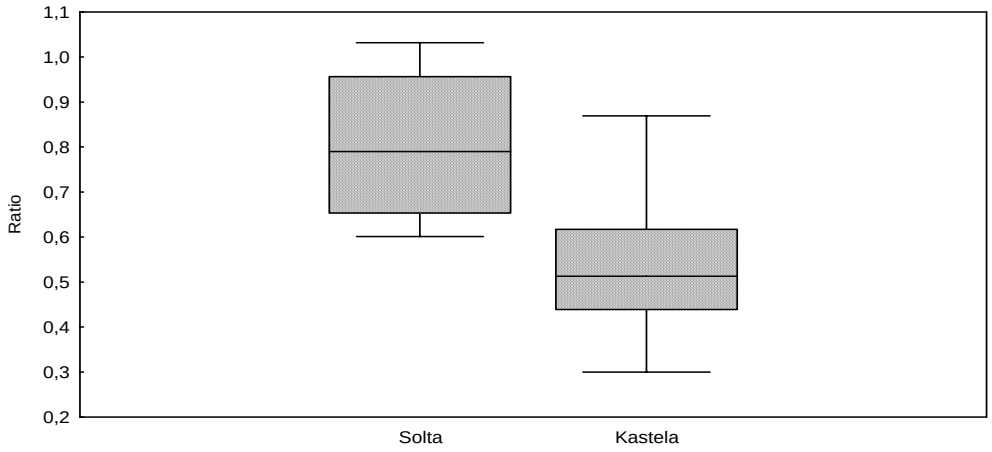

Figure 11: The ratio of Rhodamine B fluorescence measurements made on sea cucumber (Holothuria tubulosa) cells taken from individuals from station Šolta (n=10) and station Kaštela (n=5) without Pgp-inhibitor present and fluorescence measurements with Pgp-inhibotor present, respectively. Lines are median fluorescence values, boxes are quartiles and whiskers are 10 and 90 percentiles.

Discussion

The objective of this study was to develop non-invasive biomonitoring techniques, using coelomocytes from echinoderms and hemocytes from bivalve mollusks as

model and to adapt cell-based bioassays with MXR-activity and cell viability as endpoints.

Concentration of cells

Hemocytes have been found to proliferate upon infection (Arala-Chaves & Sequeira, 2000). Different pollutants have been known to act cytotoxic, and the hemocytes will undergo necrosis or apoptosis (Sokolova et al., 2004). In the hemolymph samples there were significant differences between the stations in hemocyte concentration. The concentration did not correlate with expected pollution load or mussel size. Data for size and hemocyte concentration did not show any significant correlation, which is in accordance with previous studies (Carballal et al., 1998).

Cell viability measures

Viability measurements of hemocytes from *Mytilus galloprovincialis* showed significant differences. The animals collected from Šolta had a significant higher metabolic activity than the more contaminated sampling sites. This could be due higher degree of environmental stress or lowered metabolic activity in hemocytes from the contaminated areas as enzyme inhibition or increased mortality among hemocytes. Enhanced metabolic activity has been shown in *M. edulis* after exposure to cadmium and in anoxic condition (Zwaan et al., 1995). Other studies have shown decreased metabolic activity after exposure to metals in *Anodonta cygnea* (Mouraa et al., 2000) and decreased phagocyte activity (Cajaraville et al., 1996). Apoptosis in hemocytes are suggested due to inhibition of ATPase and/or mitochondrial ADP/ATP or substrate transport (Sokolova et al., 2004). The results indicates that metabolic integrity might be a sensitive measure in hemocytes as biomarkers as there were significant differences between the non-polluted area and polluted areas. The membrane integrity was lower at the Trogir station only, and there were no significant differences between mussels collected at Šolta and mussels collected at the other stations. At Trogir the mussels were caged and there was high mortality among the group. Chemical analysis has earlier shown high concentration of contaminants at this site. The mortality could be due to lethal concentration of these contaminants. The lower integrity did not relate to a lower metabolic activity compared with the other polluted areas. The lack of lowered metabolism compared to the other polluted areas might be caused by programmed cell death, apoptosis.

Apoptosis is an energy-dependent process. Another possibility is higher hemocyte concentration in the hemocytes. This possibility was rejected by the cell counting. Membrane integrity has been shown to be disrupted in *M. edulis* after exposure to copper (Brown et al., 2004). Exposure to high concentration of chemicals may result in increased lysosomal destabilization (Hwang et al., 2002).

Both alamar Blue fluorescence and CFDA-AM fluorescence in coelomocytes was higher at the Kaštela sampling station than in the reference site Šolta. CFDA-AM fluorescence is a result of esterase-catalysed biotransformation. It is usually interpreted as the membrane stability of the cells. In this case, the assumption that esterases are in surplus to the amount of CFDA-AM added in the assay is made. This would imply higher membrane stability in the cells from the contaminated site. There are certain possible mechanisms that could cause such an increase in membrane stability, i.e., elevated levels of membrane stabilising factors, such as HSP, estrogenic compounds, cholesterol, Vitamin E or cellular or external antioxidants, Generally, the opposite results have been found with a reduction of membrane stability upon environmental stress measured in different assays (Schirmer et al., 2001). Similarly, alamar Blue fluorescence has been interpreted as a measure of metabolic activity (Goegan et al., 1995). When fluorescence measurements are interpreted as metabolic activity and membrane integrity, respectively, and the two taken together as a measure of cell viability, one gets the unexpected result of higher cell viability in cells from the contaminated site. A feasible explanation is that the Šolta individuals experienced more stress during sampling. The trawling period was somewhat longer at this station, approximately 40 minutes at the Šolta station and approximately 20 minutes at the Kaštela station. The stress was further leading to cell mortality during the incubation period and therefore decreasing the total response in each sample. This would also explain the results of Rhodamine B fluorescence, which also showed higher levels in Kaštela. Alternatively one could imagine a genetic adaptation of the population of Kastela to higher environmental stress, with cells that in some respect are more hardy to the strain of the handling in the assay. This could for instance result in a high detoxification activity in these cells. However, the sea cucumber larvae are part of the pelagic plankton, and disperse outside the parent range. The adult sea cucumber populations in Kaštela and Šolta are most likely not genetically isolated from each other, because of the pelagic larval stage. And again, this would be in contrast to

results from previous studies, where environmental stress has been found to decrease cell viability in other models.

MXR-activity

In hemocytes the level of accumulated Rhodamine 123 was highest in the hemocytes from reference site, indicating a low MXR activity. The lower accumulated substrate at the other sites could be due to a higher MXR activity which removes the substrate from the hemocytes. If there was lowered membrane integrity at these sites, the lowered accumulation could be a consequence of substrate leaching out because of membrane disruption. Only at Trogir there was significant lowered membrane integrity. As no lowered membrane integrity was detected in this assay, there is more likely to be a higher MXR activity at these sites. A higher MXR activity could be due to genetic adaptation among the mussels at polluted areas with higher levels of these proteins, or induction as a result of pollution. Also blue mussels have a pelagic larval stage which might exclude the possibility of genetic adaptation. Several studies have shown a higher degree of MXR activity in animals living in polluted areas. In transplant experiments a change in MXR activity has been shown after the moving of animals from polluted to unpolluted areas (Keppler & Ringwood, 2001; Smital et al., 2003; Smital et al., 2000).

Sea cucumber cells had a cyclosporine A sensitive ability to extrude Rhodamine B. To the knowledge of these authors, this is the first time MXR-activity is shown for *H. tubulosa*, or for any holothurians. MXR-activity measurements in coelomocytes showed a significantly higher MXR-activity in cells from individuals sampled at the Kastela station, compared to individuals sampled at the Solta station. These results are in accordance with studies on other models, showing induction of MXR-activity in individuals at polluted sites. Notably, the Rhodamine B fluorescence was higher for the Kastela samples when the protein pump was inhibited by Cyclosporin A, indicating a higher intracellular concentration of Rhodamine B for these cells. This could be due to a lower activity of drug transporting membrane proteins other than the one inhibited by Cyclosporin A. The presence of several different such proteins are known from other species, through transport-studies, immunolabeling studies etc. This would imply that in this model system one (or several) MXR-active protein in the sea cucumbers was inhibited at the same time as others were induced

(as shown by the ratio (R)) in this study. Another option is that cells from Kastela had higher cell viability than the Solta cells, which is supported by the cell viability assay. Cell death during the incubation period of the assay, would lead to lower total intracellular Rhodamine concentrations at the end. Such cell death would be expected to influence both the fluorescence with MXR-inhibitor and without MXR-inhibitor similarly, leaving the ratio of these two measurements as an applicable measure of MXR-activity in the cells.

MXR compared to cell viability

Significant differences between groups were found for all biomarkers measured in this study, i.e. differences in membrane integrity, metabolic activity and MXR activity in hemocytes from *M. galloprovincialis* originating from different stations and in coelomocytes from *H. tubulosa* originating from different stations. In hemocytes the metabolic activity was a sensitive measure, and showed large variation between the different stations. MXR activity also differed as predicted according to presumed pollution load. The membrane integrity did not differ to the same extent as the two other parameters, but was useful when considering the MXR activity in the same hemocytes. Of the parameters measured in sea cucumbers, membrane integrity showed the largest difference between locations, with the activity at Kaštela being 10-fold the activity at Šolta. Metabolic activity at Kaštela was 2.5-fold the Šolta activity, and MXR-activity was 0.5 times higher at Kaštela than at Šolta.

Conclusions

Blue mussel hemocyte viability can be impaired by contamination with a lowered metabolic activity at contaminates sites. Membrane integrity was lower only at a very contaminated site, in which several animals died. There was significant higher MXR activity at polluted sites than the reference. The sea cucumber *Holothuria tubulosa* possesses a MXR-mechanism, and the MXR-activity can be induced by contamination. The cell viability was lower in cells from the reference site; this was probably due to the sampling technique.

MXR-activity holds promise as a possible biomarker of exposure. High MXR-activity is not in it self a deleterious effect and it should therefore not be used as a biomarker of effect. The mechanisms of induction and inhibition of MXR are not fully understood, nor are the transport-proteins characterised in these species. These points should be clarified before MXR-activity is implemented as a biomarker. With the non-consistent results for cell viability found in this study, there is reason to call for caution when applying this method in future studies. However, the importance of measuring several parameters simultaneously is emphasised, as this increases the understanding of the model system as a whole.

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Vedlegg

Kjemikalier og utstyr

Biosource

alamar Blue ^{TM} (Lot 144484SA)

BioWhittaker Inc

500 ml L-15

5 ml L-glutamine

4,5 ml Penicillin streptomycin fungocon

2,5 ml $NaHCO_3$

Cayman Chemical

MK 571

Chiron
1, 2, 5, 6, 9, 10-hexabromosyklododekan (HBCD) batch no. 4076

Tetrabromobisfenol A (TBBPA)

Fluka Chemicals
Cyclosporin A (CA)

Merck
$Na_2HPO_4 \cdot 12\ H_2O$

$NaH_2PO_4 \cdot 12\ H_2O$

NaCl

Molecular probes
5-carboxyfluorescein diacetat acetoxymetyl ester (CFDA-AM) Molecular

Probes

Rathburns Chemicals

Aceton

SigmaAldrich

Tri-n-Butyltin cyanide (TBT)

Heptadecafluorooctanesulfonic acid tetra ethylamonium salt – (PFOS) CAS

56773-42

Triton X-100

Verapamil

Rhodamine B (05009EB)

Fenol

Heptadekafluorononа syre (PFNA) CAS 375-95-1

Rhodamin 123

Utstyr

Multisizer ™ 3, Coulter counter®, Beckman Coulter

CytoFluor ™ 2300, Millipore

Fluorlite ™ 1000, Dynatech Technologies

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