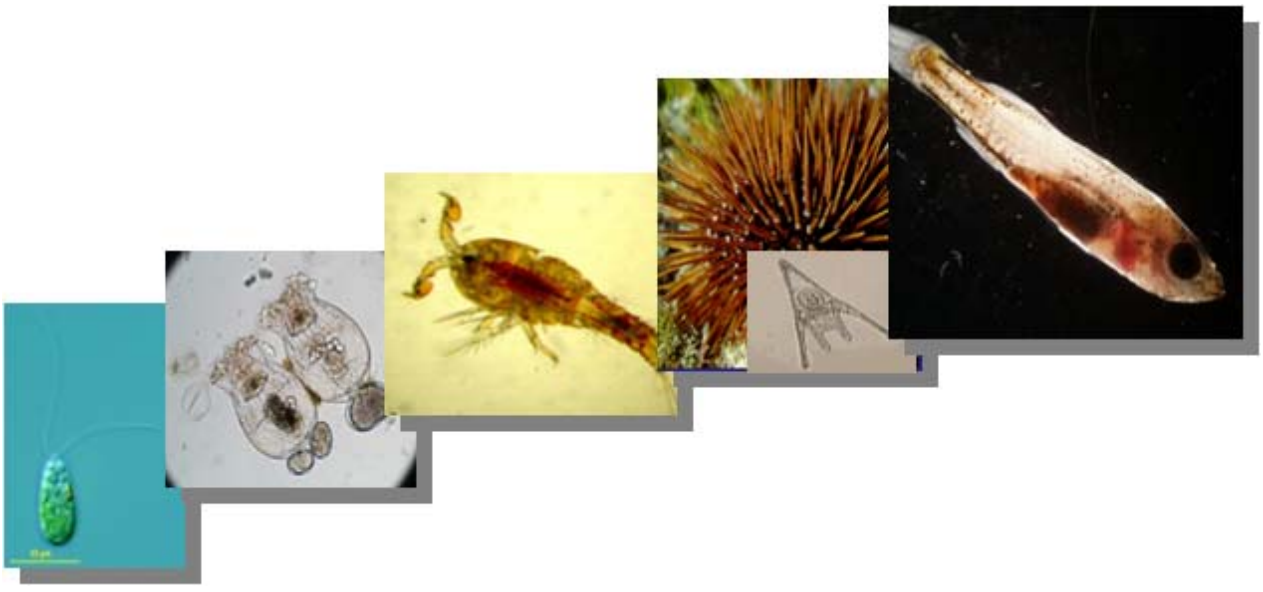
SESSIONE 1

ORGANISMI TEST E BIOSAGGI



Risultati di due interconfronti internazionali sui saggi di tossicità acuta con *Thamnocephalus platyurus* e di tossicità subcronica con *Heterocypris incongruens*

R. Baudo
CNR – Istituto per lo Studio degli Ecosistemi, Verbania – r.baudo@ise.cnr.it

Riassunto – Verranno illustrati i risultati di due confronti interlaboratorio, organizzati per validare due nuovi saggi ecotossicologici proposti per la standardizzazione in ambito ISO.

Parole chiave: tossicità acuta, tossicità subcronica

INTRODUZIONE

Gli ecotossicologi sono costantemente alla ricerca di nuovi saggi "migliorativi", che consentano cioè di ottenere informazioni più realistiche ed ecologicamente più rilevanti, possibilmente con un apprezzabile e concomitante risparmio di tempo e denaro. In quest'ottica si configura l'iniziativa della ISO (International Organization for Standardization) di sviluppare due nuove norme, attualmente in votazione per l'adozione come ISO Standard: ISO/CD 14380, Water quality - Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca) e ISO/CD 14371, Water quality - Determination of fresh water sediment subchronic toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda).

Entrambi i protocolli prevedono la possibilità che vengano utilizzati anche gli appositi ToxKit della MicroBioTests Inc., (Mariakerke, Belgium), in quanto i nuovi saggi proposti utilizzano forme durature degli organismi (il che elimina la necessità di mantenere un allevamento degli organismi).

In sintesi, il saggio con *Thamnocephalus platyurus* prevede che neonati, ottenuti dalla schiusa in condizioni controllate di uova durature (cisti) del crostaceo anostraco di acqua dolce, vengano esposti a varie concentrazioni del campione da saggiare in piastre multipozzetto (3 repliche, ognuna con 10 organismi, per ciascuna concentrazione, più un controllo negativo, pure in triplo). La piastra multipozzetto viene quindi incubata per 24 ore a (25 ± 1) °C al buio. Al termine dell'esposizione viene calcolata la percentuale di mortalità e la concentrazione letale per il 50 % degli organismi (LC50 a 24 h). Il saggio è indicato per soluzioni (o dispersioni stabili) di prodotti chimici, effluenti industriali od urbani, acque dolci, estratti acquosi e tossine prodotte da cianobatteri (per le quali *T. platyurus* si è dimostrato particolarmente sensibile).

Il saggio con *Heterocypris incongruens*, un ostracode cosmopolita, è invece, al momento, l'unico saggio sub cronico per contatto diretto, nel quale gli organismi vengono cioè esposti direttamente ad un campione di sedimento di acqua dolce (e, per estensione, a fanghi e suoli). Il saggio viene condotto con neonati ottenuti dalla schiusa di uova durature, utilizzando 6 repliche per il sedimento da saggiare e 6 repliche per un sedimento di riferimento (controllo negativo). In ciascun pozzetto di pastre multipozzetto vengono inoculati 1.000 µl di sedimento (campione o controllo negativo), 2 ml del mezzo acquoso, 2 ml di una sospensione algale e 10 neonati. Le piastre sono quindi incubate a (25 ± 1) °C al buio per 6 giorni. Al termine dell'esposizione, si contano i sopravvissuti (per determinare la percentuale di mortalità) e si confronta la loro lunghezza con quella dei neonati prima dell'esposizione. In tal modo, è possibile calcolare la percentuale di inibizione della crescita nel campione di sedimento, rispetto a quella nel sedimento di riferimento.

Secondo la politica di ISO, per essere standardizzato un nuovo saggio dovrebbe preferibilmente essere validato attraverso l'esecuzione di un confronto internazionale interlaboratorio. Pertanto, per entrambi i nuovi saggi proposti, nel 2010 sono stati organizzati tali interconfronti, in accordo con le regole ISO per la determinazione dell'accuratezza dei metodi di misura e dei risultati (ISO 5725-2: 2002).

RISULTATI DELL'INTERCONFRONTO CON *T. PLATYHURUS*

23 laboratori di 14 Nazioni diverse (Belgio, Olanda, Estonia, Ungheria, Polonia, Lituania, Portogallo, Repubblica Ceca, Repubblica Slovacca, Italia, Grecia, Canada, Guatemala e Corea del Sud) hanno aderito all'iniziativa ed hanno effettuato il saggio, secondo il protocollo previsto dalla ISO/CD 14380, per determinare la LC50 24 h del tossico di riferimento potassio dicromato, utilizzando una serie di diluizioni (0,32 - 0,18 - 0,10 - 0,056 - 0,032 mg $K_2Cr_2O_7$/L).
Tutti i laboratori sono stati in grado di effettuare correttamente il saggio, utilizzando il kit fornito dall'organizzatore e contente tutto il necessario, ad eccezione del tossico di riferimento, ed in particolare una fiala con le cisti da far schiudere per generare gli organismi da utilizzare per il saggio.
Inoltre, tutti i laboratori hanno rispettato il criterio di validità che, nel controllo negativo, la mortalità non superasse il 10 % degli organismi esposti. Anzi, complessivamente nei controlli negativi si sono registrati solo 5 morti, su un totale di 690 organismi esposti, cioè la mortalità complessiva è risultata essere pari allo 0,72 %.
Come da istruzioni, ciascun laboratorio partecipante ha quindi fornito all'organizzatore dell'interconfronto i dati relativi alla mortalità di ciascuna delle 3 repliche, tanto per il controllo negativo che per le soluzioni del tossico di riferimento.

Trattamento statistico dei dati

Tutti i dati sono stati analizzati con un apposito programma di calcolo (US EPA Benchmark Dose Software (BMDS), Version 2.1), che ha permesso di stimare, per ciascun laboratorio, il valore della LC50 e dei relativi limiti fiduciali al 95 %. Ripetibilità e riproducibilità sono state calcolate secondo la procedura indicata dalla norma ISO 5725-2 (2002), fornendo i seguenti risultati:

s_L^2 - stima della varianza interlaboratorio;

s_W^2 - stima della varianza intralaboratorio;

s_r^2 - media aritmetica delle varianze intralaboratorio dopo esclusione dei dati aberranti (outlier);

s_R^2 - stima della varianza di riproducibilità: $s_R^2 = s_L^2 + s_r^2$.

Per verificare la consistenza dei dati, sono state utilizzate le statistiche h (consistenza intralaboratorio) e k (consistenza interlaboratorio) di Mandel. Il test di Grubb è stato utilizzato per identificare straggler (dati che hanno una probabilità inferiore al 5 %, ma superiore al 1 %, di essere consistenti con gli altri dati) e outlier (dati che hanno una probabilità inferiore al 1 % di essere consistenti con gli altri dati). I risultati finali sono riassunti in tabella 1.

Tab. 1 – Valori di ripetibilità e riproducibilità calcolati per il saggio con *Thamnocephalus platyurus*.

	Tutti i dati	**Senza outlier h e k**	**Senza straggler h e k**
N. laboratori	23	22	15
Media	0,103	0,100	0,099
s_r	0,010	0,010	0,009
$C_{V,r}$, in %	9,59	9,74	9,22
s_R	0,03	0,024	0,015
$C_{V,R}$, in %	26,63	23,74	14,71
N. h straggler	0	2	0
N. h outlier	1	0	0
N. k straggler	1	1	0
N. k outlier	0	0	0
95 % UCL	0,118	0,113	0,109
95 % LCL	0,088	0,086	0,089

Secondo le regole ISO, dal set di dati si devono scartare gli outlier; pertanto, escludendo 1 outlier di tipo h, il valore medio della LC50 è pari a 0,100 mg $K_2Cr_2O_7$/L (limiti fiduciali al 95 %: 0,086 – 0,113), con uno scarto tipo della ripetibilità (variabilità intralaboratorio) di 0,010 (9,74 % come $C_{V,r}$), ed uno scarto tipo della riproducibilità (variabilità interlaboratorio) di 0,024 (23,74 % come $C_{V,R}$).

RISULTATI DELL'INTERCONFRONTO CON *H. INCONGRUENS*

26 laboratori di 14 diverse Nazioni (Belgio, Ungheria, Polonia, Portogallo, Repubblica Ceca, Italia, Svezia, Francia, Germania, Canada, Guatemala, Corea del Sud, Giappone) hanno partecipato all'interconfronto, seguendo il protocollo metodologico indicato dalla ISO/CD 14371 ed utilizzando il kit fornito dall'organizzatore che, anche in questo caso, conteneva tutto il necessario, compresa la fiala contenti le uova durature di *H. incongruens* ed una fiala con il tossico di riferimento solfato di rame ($CuSO_4.5H_2O$). Come da istruzioni, ciascun laboratorio partecipante ha quindi fornito all'organizzatore dell'interconfronto i dati di mortalità e di lunghezza dei sopravvissuti di ciascuna delle 6 repliche, tanto per il controllo negativo che per le soluzioni del tossico di riferimento.

Qualche laboratorio ha incontrato problemi nell'effettuare il saggio la prima volta ed ha quindi ripetuto il saggio una seconda volta. Per 3 laboratori, anche i risultati del secondo saggio non sono stati però ritenuti validi e quindi eliminati. Tutti i laboratori hanno comunque ottenuto una schiusa soddisfacente delle cisti e, con un'unica eccezione, hanno osservato una percentuale di mortalità superiore al 50 % per la concentrazione più elevata saggiata. Un laboratorio ha invece osservato una mortalità pari al 46,67 %, ma poiché tale risultato è sufficientemente prossimo al 50 % è stato comunque calcolato un valore di LC50 extrapolato, non utilizzato però nella successiva elaborazione statistica. Secondo il protocollo operativo, la lunghezza degli ostracodi nel controllo negativo, al termine dei 6 giorni di esposizione, avrebbe dovuto essere di almeno 400 µm superiore rispetto alla lunghezza media degli ostracodi ad inizio saggio. Effettivamente, questa condizione è stata rispettata per tutti i laboratori, tranne 4, che hanno invece misurato una crescita media finale di 353 µm, 355 µm, 367 µm e 395 µm. Le differenze sono verosimilmente da imputare a fattori ambientali, quali piccole differenze nella temperatura di incubazione, oppure a fattori biologici, come la quantità ed il valore nutrizionale delle alghe fornite all'inizio del saggio.

È stato quindi deciso di cambiare il criterio di validità: invece di richiedere un incremento fisso di almeno 400 µm, nel controllo negative, al termine del saggio, gli ostracodi devono essere cresciuti almeno 1,5 volte rispetto alla lunghezza media ad inizio saggio. Applicando questo nuovo criterio di validità, tutti i risultati risultano accettabili.

Il metodo proposto per la stima della tossicità subcronica con *H. incongruens* specifica che l'effetto subletale, cioè l'inibizione della crescita, debba essere misurato solo nel caso in cui la percentuale di mortalità nella concentrazione più elevata saggiata sia inferiore al 30 %; in effetti, non sarebbe molto significativo calcolare una inibizione della crescita se la maggio parte degli organismi risultano essere morti al termine del saggio. Le concentrazioni prescelte per l'interconfronto sono state deliberatamente individuate per avere una mortalità superiore al 50 % nella concentrazione più elevata, per consentire il calcolo di una EC50 (concentrazione che produce una mortalità del 50 %). Quindi, non rappresentano il caso ideale per stimare una inibizione della crescita (data la relativa scarsità di sopravvissuti misurabili al termine del saggio).

L'organizzatore ha comunque deciso di includere anche questo tipo di dati nell'elaborazione successiva. In 8 casi, però, l'inibizione della crescita nella soluzione più concentrata è risultata essere inferiore al 50 %, e quindi per questi laboratori non è stato possibile calcolare una EC50. In 7 casi, alla concentrazione più elevata la mortalità è risultata essere pari al 100 %, rendendo impossibile misurare la crescita. Per questi laboratori è stata allora utilizzata la crescita osservata per la concentrazione immediatamente inferiore.

Trattamento statistico dei dati

Tutti i dati, sia di mortalità che accrescimento, sono stati analizzati con il programma di calcolo US EPA Benchmark Dose Software (BMDS), Version 2.1. La successiva elaborazione, secondo la procedura indicata dalla norma ISO 5725-2 (2002) e già sinteticamente richiamata a

proposito dell'interconfronto con *T. platyhurus*, ha permesso di ottenere i risultati riportati in tabella 2.

Tab. 2 – Valori di ripetibilità e riproducibilità calcolati per i dati di mortalità del saggio con *Heterocypris incongruens*.

	Tutti i dati	**Senza outlier h e k**	**Senza straggler h e k**
N. laboratori	22	21	18
Media	5,910	5,794	5,487
s_r	0,761	0,690	0,540
CV%	12,87	11,91	9,85
s_R	1,85	1,789	1,685
CV%	31,32	30,88	30,71
h straggler	1	0	0
h outlier	0	0	0
k straggler	1	2	0
k outlier	1	0	0
95 % UCL	6,948	6,823	6,538
95 % LCL	4,872	4,766	4,436

Dopo aver escluso 1 outlier, il valore medio della LC50 è pari a 5,79 mg $CuSO_4.5H_2O$ /L, con limiti fiduciali al 95 % di 4,76 e 6,82 mg $CuSO_4.5H_2O$ /L.
Lo scarto tipo di ripetibilità S_r (variabilità intralaboratorio) è 0,69, corrispondente ad un CV% di 11,91; lo scarto tipo di ripetibilità S_R (variabilità interlaboratorio) è 1,79 (30,88 come CV%).
In tabella 3 vengono invece riportati i dati relativi all'inibizione della crescita, in base ai quali sono state calcolate le EC50, ed i relativi intervalli fiduciali, dei laboratori per i quali è stato possibile effettuare le misure di lunghezza sui sopravvissuti al termine dei 6 giorni del saggio. Escludendo 2 outlier, il valore medio della EC50 è pari a 6,58 mg $CuSO_4.5H_2O$/L (intervallo fiduciale al 95%: 5,68 – 7,48 mg $CuSO_4.5H_2O$/L), con uno scarto tipo di ripetibilità di 0,20 (3,11 come CV%) ed una scarto tipo di riproducibilità di 1,21 (18,38 CV%).

Tab. 3 – Valori di ripetibilità e riproducibilità calcolati per i dati di inibizione della crescita del saggio con *Heterocypris incongruens*.

	Tutti i dati	**Senza outlier h e k**	**Senza straggler h e k**
N. laboratori	15	13	11
Media	6,913	6,580	6,423
s_r	0,376	0,205	0,170
CV%	5,43	3,11	2,64
s_R	1,51	1,209	1,251
CV%	21,81	18,38	19,48
h straggler	1	0	0
h outlier	0	0	0
k straggler	0	1	0
k outlier	1	0	0
95 % UCL	7,949	7,477	7,440
95 % LCL	5,878	5,682	5,406

CONCLUSIONI

Il Confronto Internazionale Interlaboratorio sulla determinazione della tossicità acuta per *Thamnocephalus platyurus* (Crustacea, Anostraca), seguendo il metodo indicato da ISO/CD 14380 (2010), ha chiaramente dimostrato che:

- la schiusa delle cisti di *Thamnocephalus platyurus* dalla fiala fornita dall'organizzatore in tutti i casi ha generato un numero di organismi più che sufficiente per l'esecuzione del saggio;
- tutti i laboratori partecipanti, seguendo le istruzioni fornite, sono stati in grado di effettuare in maniera soddisfacente il saggio;
- il criterio di validità che la percentuale di mortalità nei controlli negativi non fosse superiore al 10 % è stato rispettato da tutti i laboratori partecipanti;
- l'analisi statistica dei dati di mortalità ha evidenziato un solo outlier (statistica h di Mandel); pertanto, 22 risultati, sui 23 forniti, sono stati accettati;
- lo scarto tipo medio della ripetibilità (variabilità intralaboratorio) è risultata essere inferiore al 10 %, come $C_{V,r}$;
- il valore stimato per la LC50 a 24 h ha associato uno scarto tipo di riproducibilità (variabilità interlaboratorio) pari al 23,74, come $C_{V,R}$.

Si può quindi sicuramente concludere che la determinazione della tossicità acuta per *Thamnocephalus platyurus* (Crustacea, Anostraca), seguendo il metodo delineato da ISO/CD 14380 (2010), risponda alle richieste per un saggio ecotossicologico affidabile e preciso.

Il Confronto Internazionale Interlaboratorio sulla determinazione della tossicità sub cronica su *Heterocypris incongruens* (Crustacea, Ostracoda), secondo il metodo descritto in ISO/CD 14371 (2010), ha permesso di formulare le seguenti conclusioni:

- la schiusa delle cisti di *Heterocypris incongruens* dalla fiala contenuta nel kit ha permesso in ogni caso di ottenere un numero sufficiente di organismi per effettuare il saggio;
- 23 su 26 partecipanti sono stai in grado di eseguire soddisfacentemente il saggio (anche se alcuni solo al secondo tentativo);
- il criterio di validità che la mortalità nel controllo negativo fosse non superiore al 20 % è stato rispettato da tutti i laboratori partecipanti;
- il criterio di validità di un incremento in lunghezza di almeno 400 µm negli organismi del controllo negativo non è stato rispettato in alcuni laboratori, e pertanto il criterio è stato modificato, richiedendo che al termine del saggio la lunghezza media degli organismi del controllo negativo sia almeno 1,5 volte quella degli organismi ad inizio saggio. Questo criterio è stato rispettato da tutti i laboratori partecipanti;
- per i dati di mortalità, escludendo 1 outlier il valore medio della LC50 è pari a 5,79 mg $CuSO_4.5H_2O$ /L, con limiti fiduciali al 95 % di 4,76 e 6,82 mg $CuSO_4.5H_2O$ /L; lo scarto tipo di ripetibilità S_r (variabilità intralaboratorio) è 0,69, corrispondente ad un CV% di 11,91; lo

scarto tipo di ripetibilità S_R (variabilità interlaboratorio) è 1,79 (30,88 come CV%);

- Per i dati di inibizione della crescita, calcolabili solo per 15 laboratori, escludendo 2 outlier, il valore medio della EC50 è pari a 6,58 mg $CuSO_4.5H_2O$/L (intervallo fiduciale al 95%: 5,68 – 7,48 mg $CuSO_4.5H_2O$/L), con uno scarto tipo di ripetibilità di 0,20 (3,11 come CV%) ed una scarto tipo di riproducibilità di 1,21 (18,38 CV%).

Anche per questo interconfronto si può quindi concludere che la determinazione della tossicità subcronica su *Heterocypris incongruens*, secondo il metodo ISO/CD 14371 (2010), risponde ai requisiti per un saggio ecotossicologico affidabile e preciso.

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ISO/CD 14371: 2010. Water quality - Determination of fresh water sediment subchronic toxicity to *Heterocypris incongruens* (Crustacea, Ostracoda).

ISO/CD 14380: 2010. Water quality - Determination of the acute toxicity to *Thamnocephalus platyurus* (Crustacea, Anostraca).

Valutazione ecotossicologica delle sostanze chimiche ai fini del regolamento REACH: linee cellulari stabilizzate di pesce (RTG-2)

D. Conti [a,*], S. Balzamo, C. Martone [a], M. Buchetti [a]

[a] Istituto Superiore per la Protezione e la Ricerca Ambientale ISPRA AMB LAB

Sommario – Lo sviluppo di metodi alternativi *in vitro* costituisce uno degli obiettivi del Regolamento REACH sulle sostanze chimiche. Le colture di cellule sono un sistema di facile impiego per valutazioni preliminari di composti tossici. A tale scopo vengono presentati, in seno al progetto ISPRA riguardante l'uso di linee cellulari di pesce per valutazioni ecotossicologiche, gli aspetti metodologici sviluppati in merito al mantenimento e alle caratteristiche di crescita della linea cellulare RTG-2 (Rainbow Trout Gonad).

Parole chiave: Linee cellulari di pesce, REACH, composti tossici

1. INTRODUZIONE

Uno degli obiettivi peculiari della ricerca ecotossicologica, è la valutazione degli effetti delle sostanze chimiche a livello di popolazioni ed ecosistemi che, per alcune decadi, è stata realizzata principalmente attraverso studi *in vivo*.

Tuttavia, l'entrata in vigore (1 giugno 2007) del Regolamento REACH (Registration, Evalutation and Authorisazion of Chemicals), CE n. 1907/2006, che disciplina la produzione, l'uso e l'importazione delle sostanze chimiche in ambito comunitario, ha dato un nuovo impulso allo sviluppo dei metodi alternativi *in vitro*, anche in ambito ecotossicologico. Il Regolamento REACH e le guide tecniche relative ai processi e ai metodi previsti, raccomandano che le informazioni da raccogliere in merito alla tossicità ed ecotossicità delle sostanze chimiche vengano acquisite *ricorrendo, ove possibile, a mezzi diversi dai test su animali vertebrati* (REACH art. 13), che devono quindi essere considerati come *ultima risorsa* (REACH art. 25), adottando tutte le disposizioni possibili per limitare ripetizioni inutili dei test.

La strategia delle "3R alternatives" (Russell e Burch, 1959), che fa riferimento a tutte quelle procedure che riducono (**R**educe) drasticamente il numero di animali utilizzati negli esperimenti, alleviano o minimizzano (**R**efine) le sofferenze loro inflitte e sostituiscono (**R**eplacement) in modo parziale o completo gli animali vertebrati con diverse tipologie di colture cellulari, costituisce, nell'era REACH, un impegno per tutti i ricercatori e va implementata, come indicato nel VII World Congress ALTernative to animal EXperimentation (Roma 30 agosto – 3 settembre 2009), con le R di: **R**eliable, **R**elevant, **R**eady-to-use e **R**obust, proprie dei metodi alternativi *in vitro* (Weighardt).

In particolare, gli obblighi introdotti dalla normativa REACH in merito ai saggi con specie ittiche per la valutazione di pericolosità delle sostanze chimiche riguardano: il saggio di tossicità a breve termine con pesci giovanili e con pesci nelle fasi embrionali e di avannotto; il saggio di tossicità a lungo termine con pesci adulti; il saggio di accrescimento con pesci in fase giovanile e il saggio di bioaccumulo (REACH: allegati VIII, IX e X).

I pesci sono la principale specie di vertebrati impiegata per valutazioni ecotossicologiche nella sorveglianza ambientale, nel monitoraggio e nella ricerca.

Il saggio con pesci maggiormente effettuato è quello di tossicità a breve termine che, sulla base del metodo riportato nella linea guida OCSE n° 203 (1992), richiede il sacrificio di un numero elevato (da 126 a 180) di animali per la valutazione di un solo composto chimico.

Uno dei sistemi *in vitro* proposto per ridurre l'utilizzo di pesci è rappresentato dalle cellule di pesce in coltura. In ambito ecotossicologico sono state impiegate sia colture primarie che linee cellulari stabilizzate (o continue). Queste ultime rappresentano un sistema armonizzato e di facile impiego con una variabilità relativamente bassa. Il loro uso è conveniente da un punto di vista economico e meno laborioso rispetto sia agli animali che alle colture primarie, sebbene tale sistema cellulare risulti scarsamente caratterizzato dal punto di vista delle proprietà cellulari e funzionali.

Attualmente, esistono più di 150 linee cellulari stabilizzate di pesce (Castaño et al., 2003). La maggior parte sono di tipo fibroblastico o epiteliale, crescono quindi aderenti al substrato e hanno avuto origine da tessuti di salmonidi e ciprinidi.

Le linee cellulari stabilizzate di pesce hanno alcuni indubbi vantaggi tecnici:

- alcune di esse possono essere incubate a temperatura ambiente (20°C) e in atmosfera normale (quindi non è necessario l'incubatore a CO_2 come per le cellule di mammifero);
- possono essere conservate per lunghi periodi a 4 °C per cui il congelamento in azoto liquido può essere evitato;
- possono essere esposte a varie matrici liquide con differenti osmolarità.

Da molti anni, i ricercatori propongono, per valutare la pericolosità dei composti chimici, l'uso dei saggi di citotossicità basale con cellule di pesce. Alcuni tra i metodi più frequentemente utilizzati sono: il Test del Rosso Neutro (NRU, Neutral Red Uptake) e il test MTT (Tetrazolium salt reduction assay).

Nonostante la risposta citotossica *in vitro* possa variare tra le diverse linee cellulari in funzione di differenze nell'attività metabolica o di variazioni metodologiche (composizione del siero, tempo di esposizione, temperatura d'incubazione, modalità di esecuzione dei saggi), molti studi hanno mostrato una buona correlazione con i risultati della tossicità acuta *in vivo*. In particolare, la sensibilità delle linee cellulari di pesce nel valutare la tossicità delle sostanze chimiche, è stata studiata analizzando la correlazione tra i valori di EC_{50} *in vitro* (ottenuti con i diversi saggi di citotossicità basale*)* e i valori di LC_{50} *in vivo* (ottenuti nel saggio acuto con pesci). Molti studi (Bols et al., 1985; Babich et al., 1990; Castaño et al. 1994, 2003; Lange et al., 1995; Saito et al., 1991,

* daniela.conti@isprambiente.it; tel. 0650073221; fax: 065050519

1993, 1994; Segner, 2004; Schirmer, 2006) evidenziano, per la maggior parte dei gruppi di sostanze chimiche sottoposte a valutazione, un coefficiente di correlazione r $\geq$ 0,80 (correlazione lineare positiva). Sebbene questi risultati dimostrino una comparabilità tra i sistemi *in vitro* con linee cellulari di pesce e il test acuto *in vivo* con pesci, tuttavia, in termini assoluti, i valori di EC_{50} *in vitro* sono, in media, più elevati di 1-2 ordini di grandezza, dei corrispondenti valori di LC_{50} *in vivo*. Questo significa che, per indurre un effetto tossico misurabile, le colture cellulari *in vitro* richiedono concentrazioni (nominali) di sostanza chimica più elevate rispetto a quelle necessarie nei saggi *in vivo* (Fent, 2001; Kramer, 2009).
Il problema della minore sensibilità è una peculiarità di tutti i sistemi *in vitro* in quanto tali. Infatti è stato dimostrato (Clemedson et al., 1998a, 1998b) che la sensibilità delle linee cellulari stabilizzate di pesce è confrontabile, con quella delle cellule umane e di mammifero in coltura.
Recentemente, diversi autori hanno studiato alcune modalità per aumentare la sensibilità dei saggi di citotossicità con cellule di pesce, attraverso: i) la sostituzione/riduzione della percentuale di Siero Fetale Bovino addizionato al terreno di crescita (Teneva et al., 2003; Schirmer et al., 2004; Dayeh et al., 2005); ii) la valutazione della concentrazione effettiva di composto chimico che viene a contatto con le cellule (Gülden and Seibert, 1997; 2005; 2007; iii) la selezione di *endpoint* citotossici appropriati (Schirmer et al., 2000; Shuilleabhain et al., 2004; Davoren et al., 2005; Jos et al., 2005, 2009; Zurita et al., 2007); iv) l'utilizzo di batterie di saggi di citotossicità; v) l'uso combinato di diverse linee cellulari di pesce che rappresentino i vari siti bersaglio (fegato, gonadi, branchie, cervello) dell'animale (Knauer et al., 2007; Kramer et al., 2009).
In linea con le indicazioni del Regolamento REACH in merito alla promozione dei metodi alternativi *in vitro* e con gli adempimenti previsti per ISPRA dal DM 22 novembre 2007, il Servizio di Metrologia Ambientale di ISPRA, ha avviato un progetto (approvato dal Comitato Tecnico di Coordinamento REACH), triennale (2010-2012), dal titolo: "Applicazione e armonizzazione di metodi *in vitro* per valutazioni ecotossicologiche delle sostanze chimiche ai fini del Regolamento REACH: utilizzo della linea cellulare stabilizzata di pesce RTG-2 (Rainbow Trout Gonad)".
Scopo del suddetto progetto è la diffusione presso i laboratori delle Agenzie Regionali e Provinciali per la Protezione dell'Ambiente (ARPA/APPA) dei saggi di citotossicità basale con linee cellulari stabilizzate di pesce, in alternativa al saggio di tossicità a breve termine con pesci (Conti et al., 2009). Vengono presentate in questa comunicazione le acquisizioni fin qui maturate in merito al mantenimento e alle caratteristiche di crescita della linea cellulare RTG-2.

2. Linea cellulare RTG-2

La linea cellulare RTG-2 è stata originariamente sviluppata dalle gonadi di trota arcobaleno (*Oncorhynchus mykiss*) allo stadio giovanile, per studi di virologia ittica (Wolf and Quimby, 1962).
La linea utilizzata dai laboratori di Metrologia Ambientale di ISPRA, è stata acquistata presso il Centro di Referenza Nazionale Substrati Cellulari dell'Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna (IZSLER) "Bruno Ubertini", sotto forma di fiala congelata da 1 ml (n° di passaggio: 88).
Le cellule RTG-2 sono di tipo fibroblastico e crescono come monostrato (fig. 1) adeso ad un substrato costituito da fiasche per colture cellulari con tappo a vite normale.

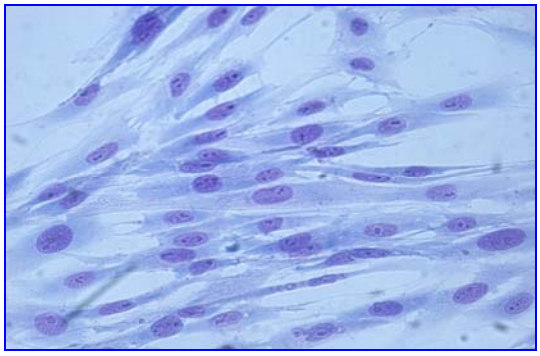

Figura 1- Monostrato di cellule RTG-2 in coltura (ingrandimento 40 X).

Le cellule RTG-2 possono sopportare ampie variazioni di temperatura (4 - 26 °C), e possono crescere con diverse formulazioni di terreno in funzione delle modalità d'incubazione delle cellule.
Per studiare le condizioni ottimali di crescita (terreno e siero), la durata della fase di latenza (lag) e il Population Doubling Time (PDT) della linea cellulare RTG-2, incubata in atmosfera normale, sono state effettuate numerose curve di crescita con differenti formulazioni di terreno e concentrazioni di siero, utilizzando diversi tipi di contenitori (fiasche, e piastre multipozzetto da 6, 12 e 24). Alcune di queste sono mostrate nelle figure da 2 a 6.
Sulla base delle prove eseguite fino a questo momento, è stato osservato che la linea cellulare RTG-2 cresce bene sia con il terreno Eagle Minimum Essential Medium (E-MEM) con sali di Earle che con quello contenente sali di Hank's (Fig. 1 e Fig. 5), sebbene questa seconda formulazione dovrebbe essere favorita nel caso di un'incubazione in atmosfera normale.
La riduzione al 5% della concentrazione di Siero Fetale Bovino (FBS) (fig. 4) o l'utilizzo di un siero più economico come il Newborn Calf Serum (NCS) (fig 6) rallenta la crescita delle cellule RTG-2, come risulta dai valori del PDT.
La combinazione di antibiotici Penicillina-Streptomicina (P/S) si è rivelata più efficace della Neomicina solfato consigliata da alcuni autori (Castaño et al., 2003).
Per quanto riguarda il contenitore di crescita, prove addizionali a quelle mostrate in questo articolo, hanno dimostrato che la linea cellulare RTG-2 cresce in modo ottimale su tutte le tipologie di plastiche (polistirene) non trattate, per colture cellulari.
Le condizioni di crescita e mantenimento della linea cellulare RTG-2 possono essere quindi così sintetizzate:
Incubazione: 20 ± 2 °C; atmosfera normale
Terreno di crescita: E-MEM con sali di Hank's o sali di Earle contenente L-glutammina e Aminoacidi non essenziali (NEAA), senza bicarbonato di sodio e addizionato con FBS al 10 % e P/S 50 UI/ml - 50 µg/ml.
Densità di semina iniziale (fiasca da 75 cm^2): 160000 cellule/cm^2
PDT medio: 2,5 – 3 giorni
Durata fase lag: 2 - 3 giorni

Frequenza dei passaggi: ogni 3 gg
Split ratio: 1:2

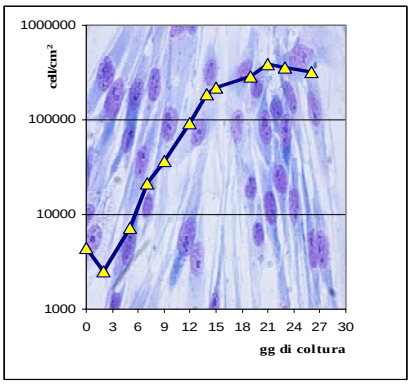

Fig. 2 – Curva di crescita 1A

Terreno: E-MEM (sali di Earle) senza sodio bicarbonato, siero FBS 10%, Neomicina solfato 5g/L; Contenitori: fiasche da 25 cm^2; PDT: 2,1 giorni

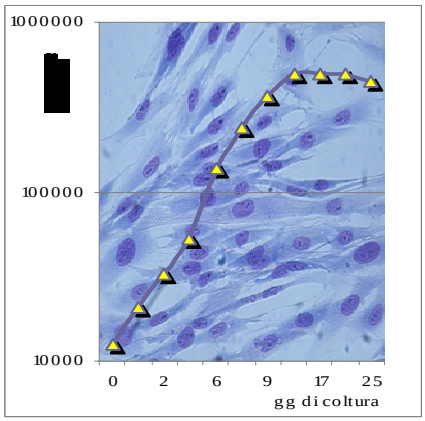

Fig. 3 - Curva di crescita 2A

Terreno: E-MEM (sali di Earle) senza sodio bicarbonato, siero FBS 10%, Neomicina solfato 5g/L, NEAA 1%; Contenitori: piastre multipozzetto 24; PDT: 2,1 giorni

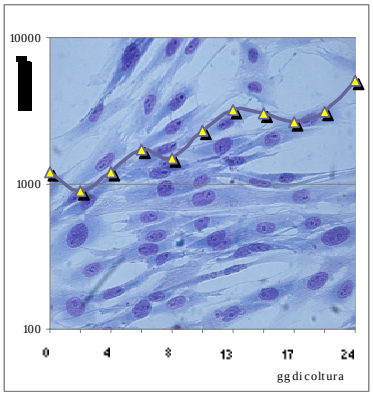

Fig. 4 - Curva di crescita 3A

Terreno: E-MEM (sali di Earle) senza sodio bicarbonato, siero FBS 5%, Neomicina solfato 5g/L, NEAA 1%; Contenitori: Fiasche da 25 cm^2; PDT: non calcolabile

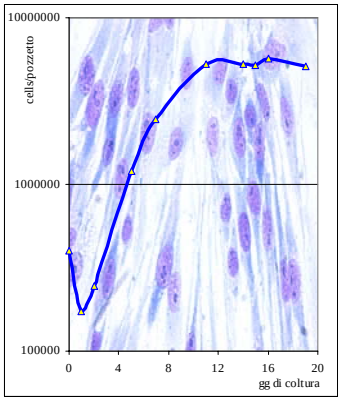

Fig. 5 – Curva di crescita 1B

Terreno: E-MEM (con sali di Hank's, NEAA e L-Glu) senza sodio bicarbonato, siero FBS 10%, P/S 50UI/ 50µg/ml; Contenitori: Piastre multipozzetto da 24; PDT: 2,5 giorni

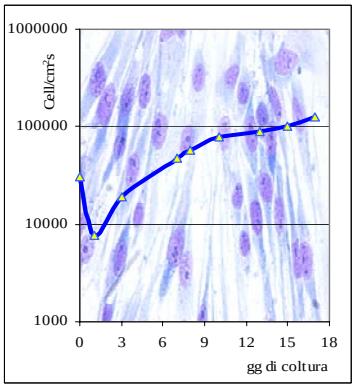

Fig. 6 - Curva di crescita 2B

Terreno: E-MEM (con sali di Hanks, NEAA e L-Glu) senza sodio bicarbonato, siero NCS 10%, P/S 50 UI/ 50µg/ml; Contenitori: Piastre multipozzetto da 24; PDT: 6,7 giorni

Una delle ragioni che hanno portato alla scelta della linea cellulare RTG-2 come modello attraverso il quale diffondere le metodiche *in vitro* ai laboratori delle Agenzie ARPA/APPA è la particolarità di questa linea di poter essere conservata in frigo a 4°C fino a due anni, senza effettuare cambi di terreno (Wolf and Quimby, 1969). Numerose prove di laboratorio eseguite da ISPRA hanno permesso di definire le condizioni di conservazione a 4°C per le cellule RTG-2, non specificate dalla letteratura, fino ad 1 anno di conservazione.

Fiasche di cellule a confluenza devono essere completamente riempite di terreno di crescita completo e poste in frigo alla temperatura di 4 ± 1°C. La presenza di siero, sia normale che inattivato (56°C per 30 minuti), è indispensabile per garantire un'idonea conservazione delle cellule. Al momento del riutilizzo le cellule devono essere staccate e riseminate in terreno completo. La linea RTG-2 rimessa in coltura, subisce dopo il primo passaggio una perdita cellulare pari al 50%, ma recupera al 2° passaggio, tornando al PDT caratteristico delle proprie condizioni di crescita.

Per cercare di affrontare il problema della minore sensibilità del sistema cellulare rispetto al saggio *in vivo*, sono attualmente allo studio sia terreni che funzionano a ridotto contenuto di siero (3% invece di 10%) che terreni *serum-free*.

3. Il Progetto per il REACH

La corretta applicazione del Regolamento REACH prevede l'organizzazione di un sistema di controllo e vigilanza, che nel nostro Paese è stato adeguato di recente, attraverso l'Accordo tra Stato, Regioni e Province autonome di Trento e Bolzano (29 ottobre 2009, G.U. del 7 dicembre 2009). Le regioni e province autonome, nell'ambito della propria organizzazione e legislazione, hanno il compito di individuare l'Autorità per i controlli sul REACH, e di nominare i propri esperti per il "Gruppo tecnico", provenienti dai ruoli delle medesime Regioni/ Province o delle Aziende Sanitarie Locali, ASL, o delle Agenzie Regionali/Provinciali per la Protezione Ambientale, ARPA/APPA. Viene inoltre stabilito che le regioni e le province autonome, debbano individuare, sulla base delle strutture analitiche già esistenti, i laboratori che eseguiranno le analisi chimiche e i saggi tossicologici ed ecotossicologici sui campioni prelevati durante le attività di controllo. Da questo punto di vista, il progetto REACH, portato avanti dal Servizio di Metrologia Ambientale, si prefigge lo scopo di promuovere e diffondere presso i laboratori ARPA/APPA le colture cellulari di pesce il cui uso, oltre ad aprire possibilità di rinnovamento scientifico, potrebbe alleggerire l'impegno richiesto alle Agenzie che si troveranno a dover eseguire i saggi ecotossicologici, in particolare il saggio acuto *in vivo* con pesci.
Il progetto prevede anche un programma di formazione da realizzare attraverso pubblicazioni, tirocinii teorico-pratici per le ARPA direttamente coinvolte nel progetto (ARPA Veneto, ARPA Sicilia, ARPA Marche, ARPA Campania) presso i laboratori ISPRA di Castel Romano e attraverso e-learning per tutte le altre Agenzie che ne faranno richiesta. E' in uscita la prima pubblicazione prevista dal progetto, dal titolo: "Uso di colture cellulari per la valutazione ecotossicologica delle sostanze chimiche ai fini del Regolamento REACH: manuale per il mantenimento della linea stabilizzata di pesce RTG-2 (Rainbow Trout Gonad)" nella collana Manuali e Linee Guida ISPRA 59/2010.

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Improved Detection of Pesticides Using a Combined SPE-Luminescent Bacteria Test

Jaap van den Dries, microLAN B.V.
M.R. Cardente, Ecotox
Supervisor: Dr. C.A.M. Broers

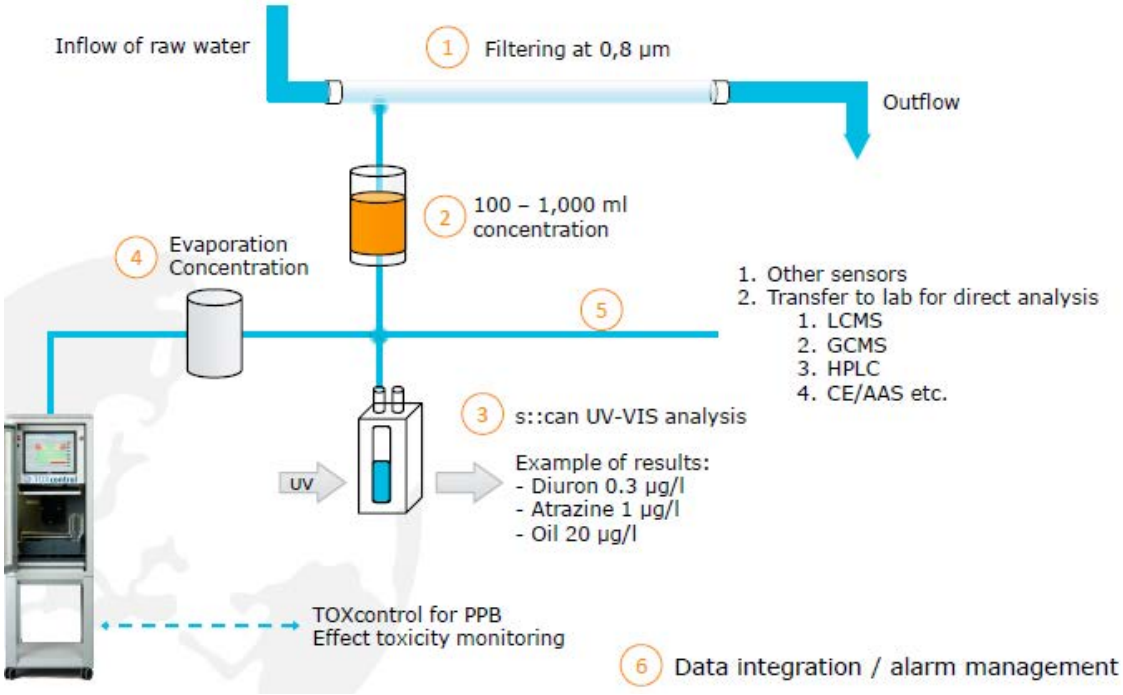
The use of early warning technologies to provide real-time information on water quality will increase in the following years for water protection against contamination. Biological sensors as alarm tools are largely applied for the application of Water Safety Plans in drinking water services (USEPA Water Security Research Action Plan) or monitoring effluents from sanitation devices discharging into water bodies (Water Framework Directive 2000/60/EC).

Main objective of the work is to evaluate a methodology for on-line monitoring of surface water quality in the intake of Drinking Water Plant suitable to detect in the ppb levels of toxicity according to detection levels mentioned in the WFD.

For that purpose an on line biological toxicity monitor using luminescent bacteria (TOX-control) has been used in combination with an on line Solid Phase Extraction system (SPE) to detect low level of pesticides, herbicides and other organic contaminants as well as pharmaceutical compounds.

Traditionally, mainly chemical techniques are applied for these purpose but the disadvantage is they do not cover the large amount of chemicals potentially present in surface water or investigate the combined effects of substances (synergistic or antagonistic).

SPE includes a concentrator and an evaporation system in order to reduce the solvent quantity in the extract. Depending on the kind of solvent, luminescent bacteria can be affected by it but methanol, 10% final concentration, can be used or DMSO.



Conclusions:

1. The SPE-TOXcontrol is capable of detecting contaminants in the low microgram / ppb range.
2. The SPE-TOXcontrol is a real alternative to time consuming but expensive laboratory analysis
3. Without additional concentration, biomonitoring systems will not alarm in the microgram range
4. Time to raise an alarm: within 2 hours

Biochemical characterization of the Tyrrhenian seaweed *Halopithys incurva*

Ramazzotti Matteo[1,2], Niccheri Francesca[2], and Degl'Innocenti Donatella*[1,2]

[1] Consorzio per il Centro Interuniversitario di Biologi Marina ed Ecologia Applicata "Guido Bacci" – Livorno, Italy
[2] Dipartimento di Scienze Biochimiche, Università degli Studi di Firenze - Firenze, Italy

Abstract – *Halopithys incurva* is a common seaweed of the Tyrrenian sea with an habitat consisting of rocky substrates. In this study we collected in 2009 samples from the Meloria shallows (Livorno, Italy) in spring (May) and autumn (November) in order to investigate possible seasonal changes in biochemical parameters and biological activities. We found deep differences in terms of both antioxidant capacity and antimicrobial activity.

Keywords: seaweed, antioxidant, antibacterial.

1. INTRODUCTION

Algae, as photosynthetic organisms, are exposed to a combination of light and high oxygen concentration, resulting in the formation of free radicals and other oxidative reagents. Since it has been observed a substantial protection from damages in their structure at both macroscopic and microscopic level, the scientific community reached the conclusion that the protection against oxidation could reside in their natural content in antioxidant substances, both constitutive or produced under stress conditions. Indeed, macroalgae are particularly rich in natural antioxidants and most of past studies identified phlorotannins as playing essential roles in the photoprotection.

Halopithys incurva (Rhodophyta, Ceramiales, Rhodomelaceae) is a small filamentous red seaweed (< 1 m) widely represented in most marine environments worldwide and in particular in the Mediterranean sea (see www.algaebase.org for a detailed distribution survey). Its habitat consists of rocky substrates in calm sunny areas with non necessarily warm waters. It can be found at depth ranging from a few cm to up to about 10 m.

In the past *H. incurva* has been investigated in terms of adaptive response to seasonal changes (Talarico and Maranzana, 2000) and, importantly, for its halogenated secondary metabolites, especially bromophenols, mainly with antimicrobial and antioxidant properties (Chantraine *et al.* 1973, Kladi *et al.* 2004). In May and November 2009 we collected samples of *H. incurva* from Meloria shallows (Livorno, Italy), at a depth of 5-10 m in order to investigate possible changes in biochemical parameters and biological activities in these two seasons so different in terms of water temperature, sun exposition and habitat in general.

* Corresponding author Donatella Degl'Innocenti,
donatella.deglinnocenti@unifi.it,
Dipartimento di Scienze Biochimiche
viale Moragni 50, 50134, Firenze, Italy
Tel. +39-055-4598302 Fax +39-055-4598905

2. METHODS

2.1 Extraction

Sample identification and collection was performed in 2009 by Dr.Luigi Piazzi. Seaweeds were washed with distilled water and dried overnight in oven at 50°C. A quantity of 4 g of dry seaweed was ground and suspended in 20 ml of 70% ethanol. Extraction was performed in two steps, the first overnight at room temperature, under continuous agitation and the second at room temperature for 6 h. The two extract were combined and defatted with *n*-hexane (ratio 1:1). The ethanol solution was finally dried using a vacuum concentrator (Univapo). Dried extracts were resuspended in 10 ml bidistilled water and used directly for further assays.

2.2 Total polyphenol content

The total phenolic (TP) content of the seaweed extract was determined according to the Folin-Ciocalteau method (Singleton V.L. et al. 1965) with some modifications. Briefly, a sample (10 µl) of the freshly prepared seaweed extract was added to 100 µl of 1:10 diluted Folin–Ciocalteu reagent. After incubation for 5 min, 80 µl of 7,5 % sodium carbonate solution was added. The mixture was incubated for 2 h at room temperature, then absorbance was measured at 750 nm with a microplate reader (Bio-Rad Model 550). Phenol content was determined using a linear extrapolation with a gallic acid reference (0–10 µg). Each assay was performed in duplicate.

2.3 Antioxidant activity

The antioxidant activity was estimated using the method adapted from Stookey (1970), with minor modifications. Ferrozine™ reagent was freshly prepared as follows: 10 mM Ferrozine™ was prepared in 40 mM HCl and mixed with 20 mM ferric chloride and 0.3 M acetate buffer pH 3.6 at 1:1:10 proportions. Aliquots of extracts (10 µl) was added to 200 µl of Ferrozine™ reagent and brought to a final volume of 250 µL with bidistilled water. After 4 min incubation at 37 °C, absorbance was measured at 593 nm at room temperature in a microplate reader. Antioxidant activity was determined using a linear extrapolation with ascorbic acid reference (0–50 µg).

2.4 DPPH radical-scavenging activity

The radical-scavenging activity was determined using the method developed by Brand-Williams et al. (1995) and modified by Fukumoto and Mazza (2000). In a 96-well microplate, 10 µl of seaweed extract was diluted in 95% methanol and mixed with 100 µl of the DPPH solution (0.15 mg/ml) in 95% methanol prepared daily.

The reaction was incubated for 30 minutes in the dark at room temperature, then the absorbance was read at 515 nm in a microplate reader. Radical scavenging activity was determined using a linear extrapolation with ascorbic acid reference (0–50 μg).

2.5 Carbohydrate assay

The total carbohydrate content of the seaweed extract was determined according to the phenol-sulfuric acid method (Dubois M. et al., 1956) optimized from Masuko T. (2005) with minor modifications. Briefly, 10 μl of seaweed extract was diluted with 60 μl of bidistilled water, then 150 μl of concentrated sulfuric acid was added rapidly and the mixture was shaken for 5 min. Then 30 μl of 5 % phenol solution was added and the mixture was heated for 5 min at 90 °C on a dry bath. After cooling to room temperature for 20 min, absorbance was read at 490 nm in a microplate reader. Carbohydrate content was determined using a linear extrapolation with D-glucose reference (0–50 μg). The results were expressed in terms of mean ± SE in three different experiments.

2.6 Antimicrobial activity

The screening for the antimicrobial activity of seaweed extracts was performed using the Bauer-Kirby disc diffusion method (Biemer J.J., 1973) against the gram positive *Bacillus subtilis* and the gram negative *Escherichia coli*.

Overnight culture of bacteria were grown to 0.5 OD at 600 nm, then inoculated 1:100 in LB-agar and dispensed in 10 cm sterile polystyrene plates. Seaweeds extracts were tested for polyphenol content, then 10 to 50 μg of polyphenol equivalent were prepared in 10 μl bidistilled sterile water and applied to Whatman 6 mm paper disks. Finally, the disks were placed on plates and bacteria were allowed to grow overnight at 37°C.

2.7 Statistical analysis

All the results were expressed in terms of mean ± standard deviation on the basis of three different experiments. Linear regression analysis on calibrator compounds and the corresponding extrapolations were made with Excel (Microsoft Corporation, Redmond, WA, USA). Nonlinear curve fitting was performed with Origin 6.0 (OriginLab Corporation, Northampton, MA,USA).

3. RESULTS

3.1 Extraction

Our extraction method was based on ethanol 70% followed by hexan-based deprivation of hydrophobic compounds (e.g. chlorophylls). Since we aimed at collecting a substantially hydrophilic fraction, the relatively high proportion of water in the initial extraction mixture was aimed at extracting both hydrophobic and hydrophilic compounds with respect to previous works. This procedure allowed us to obtain from 4 g of dried seaweeds about 58 mg (~ 1.5%) and 520 mg (~ 13%) of dried extract for May and November samples, respectively, indicating that, though simple, our method allows a consistent extraction of seaweed metabolites.

Table A. Hydro-alcoholic extraction summary.

Collection	Seaweed (g)	Extract (mg)	Yield (%)
May	4	58 ± 0.4	1.45 ± 0.01
November	4	520 ± 41	13.0 ± 1.02

3.2 Biochemical characterization

H. incurva extracts were tested for polyphenols and sugar content by means of Folin and phenol-sulphuric acid assays. As shown in Table A, November extract was found to be nearly tenfold more abundant in hydrosoluble compounds (13%) then May extract (1.45%). Despite this, according to Folin assay results (Table B), in November *H.incurva* has a lower proportion of polyphenols (37.34 ± 3.44 mg/g extract) with respect to May (69.35 ± 1.13 mg/g extract). This is even more pronounced when the sugar content is measured (Table C) with the phenol-sulfuric acid assay, indicating values of 315.34 ± 31.01 and 80.59 ± 3.84 for May and November extracts, respectively. Such results strongly support the idea that the two seasonal states of *H. incurva* are very different in terms of secondary metabolites, suggesting deep biochemical and ecological changes in response to climate variations.

Since polyphenols and sugars are known to act as scavenger for free radicals and can act as antioxidant in general, we tested the extracts with DPPH and Ferrozine assay. As reported in Table D and in agreement with the metabolite content reported above, we found a proportional increase of antioxidant and radical scavenging power in the November than in the May sample. This reinforces the idea that the metabolic condition of *H. incurva* in autumn is completely different than in spring.

Table B. Polyphenolic content (Folin method) in extracts expressed in terms of gallic acid equivalents and respect to dry seaweed or dry extract

Collection	On seaweed [mg/g]	On extract [mg/g]
May	1.00 ± 0.01	69.35 ± 1.13
November	4.85 ± 0.10	37.34 ± 0.79

Table C. Carbohydrate content (phenol-sulfuric acid method) in extracts expressed in terms of glucose equivalents and respect to dry seaweed or dry extract

Collection	On seaweed [mg/g]	On extract [mg/g]
May	4.57 ± 0.45	315.34 ± 31.01
November	10.48 ± 0.45	80.59 ± 3.84

Table D. Antioxidant and radical scavenging power (Ferrozine and DPPH assays, respectively) in extracts expressed in terms of ascorbic acid equivalents and dry extract.

Collection	Antioxidant [mg/g]	Antiradical [mg/g]
May	45.67 ± 3.62	137.39 ± 14.697
November	82.57 ± 0.49	24.84 ±1.82

3.4 Antimicrobial properties

Since previous literature identified a lot of brominated phenolic compounds in *H. incurva*, given their known ability of inhibiting microbial growth, we performed antibiograms with the single disk method, by using penicillin G and ampicillin as a reference and different doses (from 50 to 10 μg) of the extracts on *E.coli* and *B. subtilis* cells. We found that the effect of both sample types on the gram negative *E. coli* were substantially absent, while on the gram positive *B. subtilis* there were a net effect, with the November sample having a more pronounced antimicrobial activity than the May sample (Fig. 1).

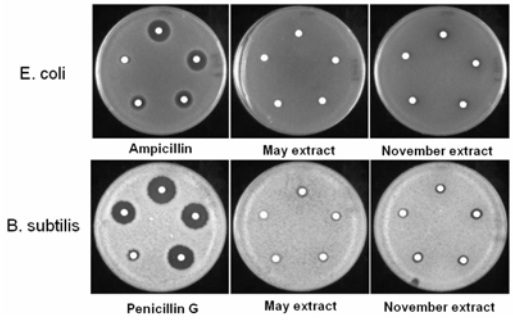


Figure 1. Antibiograms of *H.incurva* May and November extracts on *E.coli* and *B. subtilis.*

Differently to what happened with control antibiotics and in typical disc test, in our results the growth inhibition area were of poor interest, possibly due to problems in the diffusion of the antimicrobial molecules. In fact, different doses of the extracts (50 to 10 μg of polyphenols, according to the Folin assay) resulted in an inhibition area nearly double with respect to that of the disc (Fig. 2), but with different number of colonies inside reflecting different degree of antimicrobial, reasonably bacteriostatic, effect.

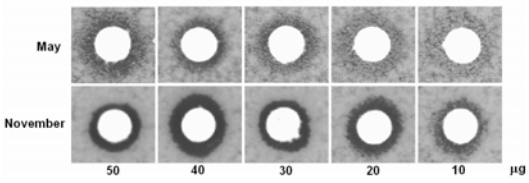


Figure 2: Detail of discs on *B. subtilis* plate for May and November extracts.

This result confirmed previous studies on antimicrobial activity of *H.incurva* extracts and evidenced a higher activity in the November extracts, despite its lower content in polyphenols with respect to the May extract. It is then reasonable to conclude that the aim of *H. incurva* secondary metabolism in November is by far more devoted at producing antimicrobial metabolites than in spring.

4. CONCLUSIONS

Our preliminary data obtained in this work support the idea that the biochemical condition of *H. incurva* in autumn is different than that in spring from several points of view, probably reflecting the different stages of its life cycle. In the future, our work will be aimed at monitoring this red seaweed in different stress condition to evaluate which are the biochemical parameters useful for monitoring its health and of marine seeweeds.

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Bioassay with the marine amphipod *Ampelisca diadema* (Crustacea): tests on liquid phase.

S. Lera [a], M. Benzi [b], P. Martini [b], C.R. Ferrari [b], P. Santini [b] ,C. Mazziotti [b,*]

[a] Istituto Superiore per la Protezione e la Ricerca Ambientale, ISPRA, Italy – s.lera@alice.it
[b] ARPA Emilia-Romagna, Struttura Oceanografica Daphne, Cesenatico, Italy – daphne@arpa.emr.it

Abstract – The use of *Ampelisca diadema* (Crustacea: Amphipoda) (Costa, 1853) as a toxicity testing organism was applied to assess the quality of whole marine sediment. In the present work, the possibility to employ the enquired species to evaluate the environmental quality of liquid phase (elutriates) was investigated. To this aim, the bioassay with *A. diadema* was carried out exposing the organisms to the elutriates for 96h, as well as the test of LC_{50} with toxicants. Collected data were compared to the results of chemical and ecotoxicological analysis on whole sediments and to standardized ecotoxicological tests on elutriates. Results shown as *A. diadema*, being an organism of benthic community and a suspension/surface-deposit feeder, can be used both in sediment and elutriate analysis.

Keywords: *A. diadema*, bioassay, elutriate.

1. INTRODUCTION

Because of its great assurance as bioindicator in assessing environmental quality, endemic species are becoming very important in the Italian ecotoxicological contest. Indeed, it has being to prefer the employment of species representatives of a particular habitants instead of species usually used to assess the environmental quality but not very representatives of the investigated contest (Calow, 1996). To this purpose, the Oceanographic Structure Daphne of Cesenatico and ISPRA Livorno, are testing an endemic Mediterranean species frequent inhabitant of muddy and sandy bottoms of the Northern Adriatic sea (Simonini et al, 2005), the marine Amphipod *Ampelisca diadema* (Costa, 1853). Due to its high sensitivity towards toxicants, its repeatability and intralaboratory reproducibility, the species can be used as indicator of pollutants in marine sediments, as found in literature (Lera *et al.*, 2007). Nowadays, this species is usually employed to carry out bioassays on whole sediment, being an organisms of the benthic community, but its feeding habitudes are very important. In fact *A. diadema* is a tubicolus, suspension/surface-deposit feeder (Grandi *et al*., 2007); for this reason it could be employed to assess liquid phase (elutriate) quality.

* Cristina Mazziotti. Arpa Emilia-Romagna, Struttura Oceanografica Daphne, V.le A. Vespucci 2, Cesenatico, Italy.
Phone number: +39054783941. E-mail: cmazziotti@arpa.emr.it

To this aim, the bioassay with *A. diadema* was carried out on different elutriates, obtained from sediments collected from various ports and their canals along the Northern Adriatic sea. On the same sediments chemical and ecotoxicological analysis had been previously conducted to investigate their quality. In this way it has been possible to verify the discriminatory capability of response of the enquired species towards elutriates. To assess the reliability of responses, the embriotoxicity bioassay with *Paracentrotus lividus* has been performed.

2. MATERIALS AND METHODS AND RESULTS

2.1 Materials and methods

The tests were performed in accordance with the standard procedures described by ISO (2005), suitably modified and adapted, in order to match with the methodologies employed by Italian authors working with the enquired species Mazziotti et al. 2010 (*in press*).

Amphipods were collected in an area located 10 km off the coast of Cesenatico, on a muddy seabed of about 13-14 m depth, using a 8-L Van Veen grab. Once collected, organisms were placed into clean plastic containers with a thin layer of sieved sediment (1-2 cm) and collection-site water. In the laboratory, organisms were maintained at a temperature of (19 ± 1°C) with a natural photoperiod, and continuously aerated.

The tests were carried out inside a 1-litre glass beakers containing different aliquots of elutriates, suitably oxygenated and varying from 250 to 300 cc. Two or three replicates per treatment with 30 individuals per replicate were carried out. Recommended size of the amphipods (2-4 mm) was previously checked. In addition, a negative control, consisting in three replicate of natural filtered seawater collected from a site far enough from anthropic pollution, were carried out. The tests were performed in static laboratory conditions. No sediment or food was added to the test chambers.

The exposure time of the organisms to the elutriates was 96h, as well as the test of LC_{50} with toxicants; indeed, in both cases, organisms are tested without sediment addition.
At the end of the bioassay, the survivors were counted. Missing amphipods were assumed to be dead. The mean of mortality value for each treatment ± standard deviation value were calculated. Mortality raw data for each treatment were compared to mortality data of negative control, and the Abbott's formula was applied.

The adopted toxicity scale is that proposed in the APAT-ICRAM manual (2007).

The sensitivity of the organisms was checked performing the 96-h LC_{50} test with atomic absorption standard cadmium solution $Cd(NO_3)_2 \cdot 4H_20$ as reference toxicant. LC_{50} value and the associated 95% confidence limits were calculated by the Trimmed-Spearmann Karber statistical method.

The following analysis had been previously performed on the whole sediments:

- Chemical: heavy metals, TBT, PAH, pesticides, PCB
- Ecotoxicological: bioassays using *Ampelisca diadema* and *Vibrio fischeri*

The following analysis had been previously performed on the elutriates:

- Ecotoxicological: bioassays using *Vibrio fischeri*

In the present work, bioassays on the elutriates were carried out at the same time performing the embriotoxicity test with *Paracentrotus lividus* and the mortality test with *Ampelisca diadema.*

2.2 Results

2.2.1 Chemical analysis

Metals, TBT, PAH, pesticides and PCB concentrations, recorded in analyzed whole sediments, are given in Table A, B and C respectively.

High concentrations of Nickel and Chrome found analyzing these sediment samples, are related to the peculiar soil composition, as previously demonstrated by studies carried out in accordance with Emilia-Romagna Region (Relation, 2008).

Table A. Heavy Metals Results on Whole Sediments

	Heavy metals (mg/Kg)						TBT
	Cd	Hg	Ni	Pb	As	Kr tot	(Sn) µg/Kg
A	0.17	0.01	54.16[a]	15.6	24.5 [a]	57.66[a]	10[a]
B	0.43[a]	0.17	65.33[a]	23.92	16.87[a]	97.65[a]	27[a]
C	0.41[a]	0.14	64.88[a]	21.42	11.7	107.3[a]	41[a]
D	0.10	0.02	34.6[a]	8.9	11.1	39.9	<5
E	0.47[a]	0.16	67.64[a]	22.31	13.77[a]	94.52[a]	41[a]
F	0.30	0.12	57.8[a]	26.1	20.8[a]	67.9[a]	5
G	<0.1	0.12	13.4	3.5	4.9	9.8	<5

[a] Values exceeding the limits indicated in the APAT-ICRAM Manual

Table B. PAH Results on Whole Sediments

	PAH (µg/Kg)							
	Benzo(a)pirene	Benzo(a)antracene	Dibenzo(ah)antracene	Pirene	Fenantrene	Crisene	Fluorantene	IPA totali
A	260.2	290.50	40.8	347.60	92.3	198.7	445.3	2626.6
B	90.3	78.00	31.4	121.50	61.6	54.9	112.1	928
C	32.9	29.10	11	58.60	30.3	23.1	49.6	392.6
D	46.5	59.30	9.4	44.80	0.4	51.6	70.9	518.7
E	82.5	78.80	24.7	83.90	40	78.8	102.6	811.9
F	352.2	336.90	58.2	389.40	167.8	228.5	490.2	3380.3
G	<0.1	0.1	0.2	<0.1	0.1	<0.1	<0.1	3.9

Yellow colored cells. Values exceeding the limits indicated in the APAT-ICRAM Manual

Table C. PCB and Pesticides Results on Whole Sediments

	Pesticides (µg/Kg)			PCB
	DDD	DDE	Esaclorobenzene	Totals PCB
A	1.6	0.6	0.25	3.82
B	0	2.5	0	9.17
C	0	2.55	0	6.49
D	0.95	0.45	1	1.44
E	0	1.9	0	2.27
F	0	1.2	0.4	6.83
G	<0.05	0.06	0.10	< 0.3

Yellow colored cells. Values exceeding the limits indicated in the APAT-ICRAM Manual

2.2.2 Ecotoxicological analysis

Results obtained by the performance of bioassays on whole sediments and elutriates are reported in Table E.

Table E. Ecotoxicological Results

	Bioassays on elutriates		Bioassays on whole sediments	
	Vibrio fischeri	*Paracentrotus lividus*	*Vibrio fischeri*	*Ampelisca diadema*
	Toxicity			
A	Low	High	Absent	Very high
B	Low	Very high	Absent	Very high
C	Low	Very high	Absent	Very high
D	Low	Absent	Absent	Very high
E	Low	Very high	Absent	Very high
F	Low	Very high	Absent	Very high
G	NA	NA	Absent	NA

NA. Not available data

Sediments samples resulted polluted if tested with the amphipod *A. diadema*, as demonstrated by the mortality data increase; on the other hand, bioassay performed using *V. fischeri* bacteria didn't show any effect.

Embriotoxicity test with *P. lividus* carried out on elutriates showed different levels of contamination, varying from unpolluted to highly polluted ones. These results are confirmed by chemical analysis, that revealed high concentrations of TBT in samples A, B, C, E and of PAH in samples A, B, E and F. Toxicity was also confirmed by a general and diffused low toxicity found with the *V. fischeri* bioassay. On the other hand, results obtained with *A. diadema*, revealed a medium pollution in the 40% of the samples and the rest didn't show any contamination (Table F).

Table F. Bioassay with *A. diadema* on elutriates

Samples	Mortality Mean ± SD	Abbott Mortality (%)	Toxicity
A	4,50 ± 0,71	15,00	Absent
B	6,50 ± 3,54	21,67	Medium
C	4,00 ± 2,83	13,33	Absent
D	2,00 ± 0,00	6,67	Absent
E	7,00 ± 0,00	23,33	Medium
F	3,50 ± 0,71	11,67	Absent
G	8,00 ± 1,41	26,67	Medium

Interesting outcomes are related to sample D. Indeed, it is the only one which resulted unpolluted for the sea urchin *P. lividus* embriotoxicity test, and it is the ones which presents the lower percentage rate of mortality with the amphipod *A. diadema* (6,67%); moreover, is the only one where heavy metals concentrations don't exceed the quality standard values accepted by the APAT-ICRAM manual and even if some PAH values exceed the limits indicated in the above mentioned manual, the same values are not so far from the limits.

4. CONCLUSION

Results obtained performing the acute mortality test with the marine amphipod *A. diadema* are comparable with those obtained performing the embriotoxicity bioassay with the standardized species *P. lividus* on the same elutriate samples. Both bioassays in fact present a discriminatory capability of response towards different levels of contamination. The other bioassays, carried out on whole sediments and/or on liquid phase, seem to give similar and unique responses and seem not to be able to discriminate between different grade of pollution. In this way, the embriotoxicity test with *P. lividus* confirm its high sensitivity and discriminatory capability towards different kind of pollutants (Chapman, 1995) and supports the use of *A. diadema* in ecotoxicological investigations concerning solid and liquid phases. Results show as the acute mortality test seems to be more sensitive when applied to whole sediment, where the toxicity levels in each sample results "very high"; on the other hand its discriminatory capability is evident only if applied to elutriates, whose toxicity varies from "absent" to "medium". Moreover, only through the performance of the bioassay on elutriates has been possible to identify the less polluted sample (sample D). Anyway, the differences among the levels of contamination indicated by the two species, suggest the necessity of specific toxicity scales, performed *ad hoc* on the employed species. Toxicity results obtained in this work for *A. diadema* come out from the same toxicity scale adopted for the amphipod *Corophium orientale*, as indicated in the manual APAT-ICRAM, but as found in literature, *A. diadema* is much more sensitive than other amphipods towards organic and inorganic toxicants (Lera *et al.*, 2007).

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Mortality prediction (apoptosis) in copepods exposed to toxic compounds

I. Buttino [a, b] *, D. Pellegrini [b], J.-S. Hwang[c], T.-M. Liu[d], C.-K. Sun[e], G. Romano [a], D. Sartori [b], A. Ianora [a]

[a] Stazione Zoologica Anton Dohrn Napoli, Italy – buttino@szn.it
[b] Istituto Superiore per la Protezione e Ricerca Ambientale ISPRA-STS Livorno, Italy
[c] Institute of Marine Biology, National Taiwan Ocean University, Keelung, Taiwan
[d] Institute of Biomedical Engineering, National Taiwan University, Taipei 10617, Taiwan
[e] Department of Electrical Engineering, National Taiwan University, Taipei, Taiwan

Abstract – External stress can induce apoptosis in cells and tissues that can be revealed by immuno-fluorescence techniques. Here we discuss recent techniques used to predict death of copepod embryos and adults, both in alive or fixed samples. The most efficient probe for alive samples is Annexin V-FITC while TUNEL technique, a non-vital fluorescent probe, was used to detect apoptosis in fixed copepods. Finally, we apply, for the first time, in vivo higher harmonic generation microscopy to analyze morphology of *Acartia tonsa* nauplii exposed to different concentrations of $NiCl_2$. Future perspectives in the use of these new technologies able to reveal apoptosis without fluorescent probes, will also be discussed.

Keywords: Programmed cell death, confocal microscopy.

INTRODUCTION

Apoptosis or programmed cell death is determined by precise genetic and molecular signals (Williams, 1991), triggered by physiological events such as metamorphosis in amphibians and fetal development in mammals, or by external factors such as exposure to toxic compounds or to environmental stress (Rastogi *et al.*, 2009). Apoptotic processes can be revealed early in cells, tissues and organs due to certain peculiar characteristic events inducing morphological and biochemical alterations (see Krysko *et al.*, 2008 for a review). Among the morphological modifications are cell shrinkage, nuclear blebbing and condensation, with formation of membrane bound-vesicles called apoptotic bodies. Alteration of DNA with consequent fragmentation and degradation and the inversion of plasma membrane permeability, coupled with the exposure of phosphatidyl serine residues to the cell surface, are other morphological events common to apoptosis in many biological systems. Specific biochemical and molecular signals begin with cascades of events including activation of cysteine-dependent proteases (caspases in mammals or cysteine protease-homologs in non-mammals) (Yuan *et al.*, 1993), release of cytochrome c from the mitochondrial inter-membrane space to the cytosol, and activation of hundreds proteins are involved, as part of an extremely fine-tuned regulatory network consisting of pro- and anti-apoptotic factors. These events differentiate apoptosis from necrosis death; the latter generally occurs when cells are exposed to extreme conditions which may result in damage to the plasma membrane, and begins with an impairment of the cell's ability to maintain homeostas is. As a consequence, an influx of water and extracellular ions leads to cell swelling and rupture (cell lysis) with the release of cytoplasmic contents into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense. inflammatory response Generally, apoptosis and necrosis follow a temporal sequence with necrosis succeeding apoptosis but often the intensity of the initial insult decides for the prevalence of one process rather than other (Behilarz *et al.*, 1995, Leist & Nicotera 1997). The complexity of events inducing apoptosis in pluricellular organisms and the significance of different modes of death, are under continuous investigation, especially in human pathology where the characteristics of cell death are considered diagnostic. Morphological observation of cells in light microscopy (time-lapse imaging using differential interference contrast microscopy DIC) is commonly used as a first screen to differentiate between apoptosis and necrosis. However, in marine invertebrates, as for example crustaceans, the presence of a thin chitinous wall surrounding embryos, nauplii and adults, render the analysis of cell morphology very difficult. Therefore, specific fluorescent probes are the only tool to study this process in marine crustaceans. In marine invertebrates, apoptosis has been detected by adapting methods developed for cultured vertebrate cells (Seipp *et al.*, 2001; Voronina & Wessel 2001). In particular, some fluorescent probes have successfully been used to detect PCD or necrosis in sea urchin embryos (Romano *et al.*, 2003), crustaceans (Buttino *et al.*, 2004) and tunicates (Tosti *et al.*, 2003).

Among the different probes, vital fluorescent markers are able to detect apoptosis or necrosis in live organisms, while non-vital markers can be used only for fixed samples.

Here we review recent progress in the study of induction and detection of apoptosis in marine zooplanktonic copepods after their exposure to different apoptotic-inducer compounds: decadienal, a diatom-derived compound, and Nickel Cloride. We propose some methods to predict copepod mortality, even when organisms appear normal and viable; these techniques could be useful in ecotoxicological studies. Moreover, new and promising microscopy

* Isabella Buttino Viale Nazario Sauro, 4 – 57128 Livorno (Italy) e-mail: isabella.buttino@isprambiente.it
Phone and Fax: +39 0586 260201

techniques, second and third harmonic generation microscopy, able to reveal apoptosis in whole-mount animals without using fluorescent probes, are also described.

MATERIALS AND METHODS

Annexin V-FITC

Annexin V-FITC (fluoresceine isothiocyanate) is a vital fluorescent probes able to bind phosphatidyl serine residues that are externalized on the plasma membrane surface during early phases of apoptosis (Aubry *et al.*, 1999; Bossy-Wetzel & Green, 2000). Therefore, cells undergoing apoptosis, show positive green fluorescence. This probe has been used to detect apoptotic embryos spawned by the copepod *Calanus helgolandicus* fed 10 days giant liposomes encapsulating with 3.6 ng ml^{-1} decadienal, a well-known apoptotic-inducer compound extracted by diatoms (Buttino *et al.*, 2008). The protocol consists in a preliminary permeabilization (30 min) of the embryonic chitinous wall with 1 Unit ml^{-1} chitinase enzyme (Buttino *et al.*, 2004). Such incubation does not interfere with normal development of embryos. After rinsing in filtered seawater, embryos were incubated for 30 min with Annexin V-FITC (Alexis Biochemicals) at a concentration of 250 µl ml^{-1}. Samples were observed with a Zeiss inverted epifluorescent microscope using 20x objectives.

TUNEL

When cells enter apoptosis, lysis of endonucleases trigger the cleavage of DNA into specific oligonucleosomic fragments that can be identified both histochemically and immunocytochemically (Iseki & Mori, 1985, Kaufmann *et al.*, 2000). In particular TUNEL reaction relies on the incorporation of labeled dUTP into DNA using terminal deoxynucleotidyl transferase TdT (TdT-mediated dUTP Nick End Labelling or TUNEL) (Gavrieli *et al.*, 1992). Excitation with 488 nm wavelength fluorescent microscope, dUTP emits green fluorescent light which allows detection of apoptotic nuclei that appear green. *Calanus heloglandicus* embryos were fixed in 4% paraformaldheyde for almost 24h but for no more than 5 days to avoid DNA damage due to formaldehyde fixative, rinsed several times in PBS and frozen in liquid nitrogen, at least three times, to fracture the carapace. For adults *Calanus helgolandicus* the head may be severed to better permeabilize whole mount specimens (Buttino *et al.*, 2008). Permeabilization with chitinase solution enzyme follows this treatment: samples were incubated for 24 h in 250 µl of 1 U ml^{-1} chitinase enzyme (EC3.2.1.14; Sigma-Aldrich) dissolved in 50 mmol l^{-1} citrate buffer, pH 6, at 25°C, to permeabilize the chitinous wall. After rinsing several times in PBS, samples were incubated for 2 h in 0.1% Triton X-100 at room temperature, rinsed in PBS containing 1% BSA, and further incubated for 90 min in TUNEL solution at 37°C. To obtain TUNEL-positive samples, embryos were incubated for 10 min in 50 mmol l–1 Tris-HCl, pH 7.5, 10 mmol l–1 MgCl2, 0.1% dithiothreitol, containing 250 mg ml–1 DNase I (grade II from bovine pancreas; Boheringer GmbH, Mannheim, Germany) at room temperature. Negative controls were obtained by incubating embryos in label solution only, as recommended by the manufacturers of the TUNEL kit (Romano *et al.*, 2003).

Second and third harmonic generation microscopy

Harmonic generation microscopy (HGM) is a laser scanning nonlinear microscopy technique which acquires optical sectioning images with submicron spatial resolution (Chen *et al.*, 2001; Sun *et al.*, 2004). Based on an infrared Cr:forsterite femtosecond laser operating around 1230 nm, HGM can achieve deep penetration depth and least damage compared with commonly used Ti:sapphire lasers (Sun *et al.*, 2004). The corresponding second harmonic generation (SHG) and third harmonic generation (THG) signals fall in visible wavelength range, making it compatible to microscope optics. To test the ability of 2PM and HGM to detect apoptosis in copepods without using any fluorescent probes, nauplii of the copepod *Acartia clausi* were incubated, soon after hatching, in a solution of $NiCl_2$ at concentrations of 0.016; 0.025 and 0.063 mg L^{-1}. After 7 days live nauplii were fixed in 4% PAF for 48h, rinsed in PBS and 0.1% sodium azide, and were sent to the Department of Electrical Engineering, National Taiwan University, Taipei (Taiwan). Samples were observed with the HG microscopy 2PM at the Research Center for Medical Excellence - Division of Genomic Medicine, National Taiwan University. The concentration of $NiCl_2$ used were below or equal to those calculated in our preliminary experiments as lethal concentration inducing mortality in 50% of hatched nauplii (LC_{50}) after 48h (0.19 to 0.25 mg L^{-1}) and after 7 days (0.038 to 0.063 mg L^{-1}) (unpublished data). The intensity of two photon red (>665nm) fluorescence images are represented with flame-like color scale, and the fluorescence level of control was amplified 100 times to show greater details.

RESULTS

When *Calanus helgolandicus* females fed for 6 days giant liposomes containing 3.6 ng ml^{-1} decadienal, less than 50% of their embryos hatched (Fig. 1A) and after 8 days most of embryos were positively stained with annexin V-FITC (Fig. 1B) suggesting that apoptosis was started and the embryos will die soon afterwards. Similarly, female via-bility decreased after 8 days of feeding and after 9 days more than 50% died (Fig. 2A). Most of live females, stained with TUNEL, showed a strong green fluorescence (Fig. 2B) indicating that apoptosis was starting and that they will die soon afterwards. TUNEL positivity suggested that apoptosis was extended to the whole body (referred as phenopthosis) (Skulachev, 1997).

Figure 3 shows *Acartia tonsa* nauplii exposed 7 days at the Nickel concentrations and observed at SHG (green) and THG (magenta) microscopy. The fluorescence level of control was amplified 100 times to show greater details. THG signals show strong signals in nauplii exposed to the highest concentration of Ni (0.063 mg L^{-1}) in contrast to a weak fluorescence of control nauplii and those exposed to a lower nickel concentrations. The THG signals, evidenced in the inner part of the gut (arrows) could be due to apoptosis-related chemicals or vesicles. On the contrary, SHG microscopy, showing muscles, give a fluorescent signal similar to those recorded for all treatments.

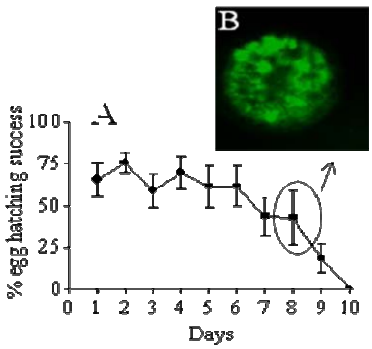

Figure 1. Hatching success of *Calanus helgolandicus* females fed for 10 days liposomes encapsulating decadienal at a concentration of 3.6 ng ml^{-1} decadienal. After 8 days more than 50% embryos were not viable (A). These embryos were also positively stained with Annexin V-FITC (B) indicating that apoptosis was starting (magnification x 200). (Modified by Buttino *et al.*, 2008).

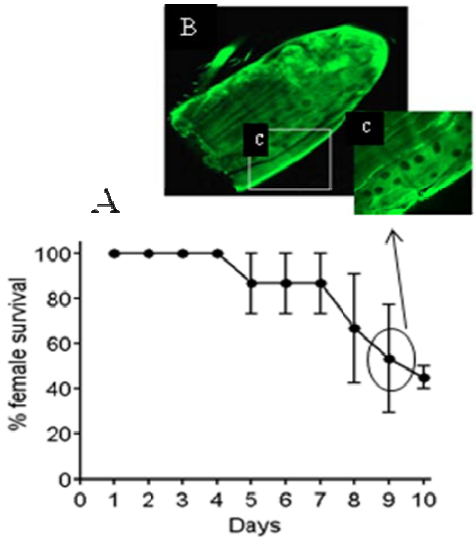

Figure 2. *Calanus helgolandicus* females fed for 10 days liposomes encapsulating decadienal at a concentration of 3.6 ng ml^{-1} decadienal. Female viability decreased to less than 50% after 9 days (A). Most of these females were positively stained with TUNEL (B) indicating that apoptosis was starting (magnification x 100). C) Magnification of the gonad in B with oocytes in dark (Magnification x 250). (Modified by Buttino *et al.*, 2008).

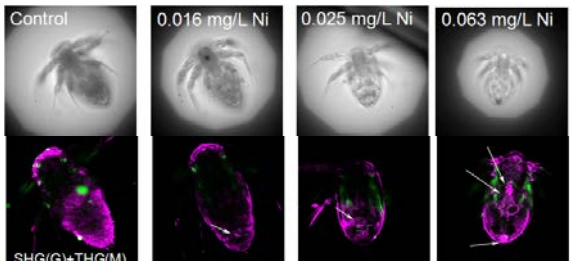

Figure 3. *Acartia tonsa* nauplii incubated in $NiCl_2$ for 7 days and observed with a light microscopy (first row) and with a combination of second (SHG) (green) and third (THG) (magenta) harmonic generation microscopy. Control nauplii shows a weak auto-fluorescence and the fluorescence output was increased 100x to visualize the structures. Nauplii exposed to 0.016; 0.025 and 0.063 mg/ml $NiCl_2$ show an increased magenta fluorescence at the region corresponding to digestive apparatus (arrows).
SHG microscopy shows muscles. (Magnification: 200x, field of view 750µm×750µm).

DISCUSSION

Fluorescent probes used in this study to visualize apoptotic cells in copepods have recently been used to predict embryo and adult viability. However, an accurate screening of programmed cell death is possible through an integrated approach based on several morphological and biochemical observations. No single technique is adequate to unequivocally demonstrate apoptosis, and different complementary methods must be applied for any individual model. The "apoptosis" concept in copepods and more in general in zooplankton is relatively new; Poulet *et al.* (2003) used a double-labelling method to stain *C. helgolandicus* nauplii with Annexin V-FITC coupled with propidium iodide (PI), to simultaneously label apoptotic and necrotic cells in live copepod nauplii. PI stains nuclei of necrotic cells which lose their plasma membrane permeability compared to non-permeant live cells. Dead cells appear with nuclei fluorescent in red (necrotic cells), whereas live cells starting an early apoptotic process, appear fluorescent in green. Moreover, vital fluorescent probes as Fluoresceine Diacetate or its homologous, Sytox green and 7-aminoactinomycin (7-AAD), have been used to discriminate between live or death cells in copepods but they were not able to differentiate between necrosis and apoptosis even if their application can be useful to rapidly detect copepod embryo viability without having to wait for eggs to hatch (Buttino *et al.*, 2003; 2004).
Among the classical non-vital fluorescent probes used to detect apoptosis, the *in situ* nick-end labelling of DNA strand breaks TUNEL, has been successfully used to stain copepod embryos, nauplii and adults (Romano *et al.*, 2003; Ianora *et al.*, 2004; Buttino *et al.*, 2008). The specificity of this technique for apoptosis is, however, under debate because some studies have reported that TUNEL does not discriminate between necrosis and apoptosis (Walker & Quirke, 2001). TUNEL staining is, for example, strongly dependent on the type and length of fixation (Negoescu *et al.*, 1996). To confirm that TUNEL staining in copepod embryos was really due to apoptosis, Romano and co-authors (2003) coupled this method with DNA laddering. The laddering of ethidium bromide-stained DNA fragments on a gel is one of the classic methods to reveal apoptosis (Earnshaw 1995). However, this technique requires a large number of embryos and cannot be recommended for routine screening of apoptosis in copepod embryos.
Results obtained also with new, non invasive second and third harmonic generation microscopy show that these techniques, applied for the first time on copepods, could be useful tools to detect apoptosis both in live or fixed copepods, without using fluorescent probes. Because the permeabilization of the chitinous wall in copepods, and more in general in crustaceans, is a serious problem, these

techniques represent an innovative approach to study their physiology and ecotoxicology. The THG microscopy can depict the boundaries of cells or the distribution of vesicles. Its signal intensity is determined by the contrast of the refractive index and a material related constant (Chu *et al.*, 2003; Sun *et al.*, 2004; Liu *et al.*, 2008). SHG microscopy has been shown to reveal the distribution and orientation of structured proteins such as collagens or microtubules in live zebrafish embryos (Sun *et al.*, 2004). This technique allows to visualize structures in *in vivo* embryos without any invasive protocols. In a recent paper, strong THG signals were found associated to the apoptotic bodies in the hind brain of zebrafish. The same apoptotic bodies were positively stained with acridine orange and detected by 2-Photon fluorescence microscopy (Chen *et al.*, 2006; Hsieh *et al.*, 2008). In our experiments THG signals increased in nauplii exposed to higher concentrations of heavy metal, suggesting apoptosis induction as demonstrated in zebrafish embryos. If this hypothesis is confirmed by further studies, this non-invasive technique can be used to predict naupliar mortality. This approach could have interesting applications for future "in vivo" studies, for example collecting organisms in some polluted areas and detecting toxicity without waiting for acute or chronic effects.

CONCLUSIONS

The identification of active programmed cell death (apoptosis) in copepods opens new and unexpected possibilities to predict embryo and naupliar viability in zooplanktonic communities. Since some apparently normal copepod embryos or deformed nauplii are positive for apoptotic markers the possibility to predict egg, naupliar or adult mortality appear realistic and this approach could be proposed in ecotoxicological studies.

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Effects of ochratoxin A on apoptosis during embryonic development of *Zebrafish.*

G. Chiarelli[1], R. Favorito, I. Ferrandino, F. Tesorone, M. Lancieri and M. Guida

Department of Biological Sciences, University of Naples "Federico II" , Napoli, Italy

- **Abstract – Ochratoxin A (OTA), a toxin produced by Aspergillus ochraceus and Penicillium verrucosum, is one of the most abundant food contaminating mycotoxins. we performed a toxicity test on zebrafish embryos at concentrations of 1 mg/L, 100 µg/L, 10 µg/L, 1 µg/L OTA. In this work we observed the induction of apoptosis by OTA in zebrafish embryos. In particular, apoptosis was evaluated as a direct result of oxidative stress and increased levels of lipid peroxidation using the system of deoxynucleotide terminal transferase (TUNEL). This method was performed on zebrafish embryos in the presence of 10 µg/L OTA. In 144 hpf embryos treated, however, has been observed that apoptotic events were more marked than the control. The results obtained by TUNEL were in agreement with the findings of recent studies linking an increase in apoptosis of ochratoxin A by the ability to induce oxidative stress and lipid peroxidation.**

Keywords: Zebrafish, Ochratoxin A, apoptosis, TUNEL.

1. INTRODUCTION

Due to the increasing use of vegetable ingredients in feed for most commercial fish species, awareness of mycotoxin problems in fish farms has increased. So there is more attention to minimize their negative impact on performance and health of fish exposed. Ochratoxins, the second major group of mycotoxins, are secondary metabolites produced primarily by Aspergillus ochraceus and related Aspergillus species, as well as Penicillium verrucosum and certain Penicillium species (CAST, 2003). Ochratoxin-A (OTA) is the major metabolite of toxicological significance, potentially as important as the aflatoxins. Chemically, OTA structure implies the linking between two moieties: a substituted dihydroisocoumarin and L-b-phenylalanine (Kuiper-Goodman and Scott, 1989). Consequently, it disturbs cellular physiology, mostly by inhibiting the enzyme involved in the synthesis of the phenylalanine-tRNA (Bunge et al., 1979; Marquardt and Frohlich, 1992), inhibiting mitochondrial ATP production (Meisner and Meisner, 1981), and induction of oxidative stress (Omar et al., 1991; Sava et al., 2006). Recently, it has been suggested that OTA mediates its toxic effects via induction of apoptosis, disruption of mitochondrial respiration and/or the cytoskeleton or via generation of DNA adducts (O'Brien and Dietrich, 2005).

The early life stage (ELS) test using zebrafish embryos is currently one of the most widely used tools for investigating the detrimental effects of aquatic pollutants in fish. Several authors consider early life stage to be the most sensitive (McKim, 1977; Eaton et al., 1978; Kristensen, 1995; Luckenbach et al., 2001), though, this may not necessarily be true for all compounds and species. The growing embryos offer many diverse endpoints to determine sublethal effects. The zebrafish, Danio rerio, is a small, freshwater, aquarium species which is easy to grow and maintain in different environments, has a short generation time, and breeds almost all year round. Much has also been written about the development (e.g. Hisaoka and Battle, 1958; Westerfield, 1998) and ecotoxicology of this species (e.g. Laale, 1977; Groth et al., 1993; Herrmann, 1993; Ensenbach and Nagel, 1997). The species could, therefore, serve as an excellent model for studying embryotoxic effects induced by pollutants.

By virtue of these data, the aim of this study was to evaluate the effects of ochratoxin A taken to concentration of 10µg/L, concentration where toxic effects are statistically significant compared to controls, during embryonic development the teleost *Danio rerio*. We also observed phenotype in vivo to assess the damage induced morphological and behavioral of OTA. In particular, we evaluated the apoptotic process as a direct result of oxidative stress and increased levels of lipid peroxidation using the system of deoxynucleotide terminal transferase.

2. MATERIALS AND METHODS

2.1 Origin and maintenance of parental fish

Danio rerio adults were obtained from a commercial dealer and were kept in 30L full glass aquaria with the following conditions: 26 ± 1 C, with a 14-h/10-h light/dark cycle. They were fed with frozen red mosquito larvae from an uncontaminated source, alternatively with commercially available artificial diet (TetraMine flakes), twice daily. On the evening before spawning was required, several rectangular mesh wire boxes were laid on the bottom of the aquaria to collect the eggs the following morning. One female spawns between 50 and 200 eggs on a daily basis. Egg production can be significantly stimulated by additional rations of natural food (Artemia spec. nauplii; Daphnia). The fish used for producing eggs should be between 4 and 15 months of age. Spawning was triggered once the light was turned on and was completed within 30 min.

2.2. Ochratoxin-A preparation

Commercial-grade OTA (Sigma chemical Co., USA) was handled with all precautionary measures, using necessary protective equipment. It 'was prepared in a stock solution of OTA concentration of 10µg of ochratoxin A in 1µl DMSO.

The stock solution was appropriately diluted and added to cultures of embryos composed of distilled water and 60µg/ml of "Instant Ocean" Sea Salts.

[1] Corresponding author. PhD Student, Tel. 081.2535030 Fax. 081.2535035 e-mail: giovanni.chiarelli@unina.it

2.3 Toxicant, exposure procedures and Detection of apoptotic cells

The eggs were collected and rinsed several times with tap water. To start exposure immediately, the eggs were transferred to various exposure chambers. At around 2–4 h postfertilization, only the fertilized eggs (blastula stage) were selected and transferred to each of the 6-well-plate (50 per plate) containing different concentrations of OTA. Detection of apoptotic cells by using the system of deoxynucleotide transferase end on treated embryos with 10µg/L OTA.

3. RESULTS

The toxicity test showed that at concentrations of 10µg/L OTA the mortality rate of embryos in 24hpf Tartt is 21% and reaches 60% 144hpf compared with controls. The observation of live embryos at different developmental stages, from the 24hpf - 144hpf showed the existence of defects, affecting primarily the trunk and head. The embryos treated with OTA at concentrations of 10µg/L had difficulties with balance and swimming, as well as alterations in the trunk, showed kyphosis, an accentuation of the normal curvature of the lumbosacral spine, abnormality that prevents embryos occur in the right anterior-posterior extension. The system of deoxynucleotide terminal transferase was used to assess that treatment with ochratoxin A could influence the mechanisms that regulate apoptosis during embryonic development of Danio rerio. Our study was performed on embryos treated with OTA 10µg/L at 24hpf and 144hpf, and the respective control embryos. In the 24hpf control embryos, apoptotic cells were founded due to the normal morphogenetic processes of the embryo. Contrary to expected, in embryos treated 24hpf observed fewer apoptotic cells in all regions considered, compared to controls, except for the central nervous system and the equipment urogenital. In control samples 144hpf at eye level and the abdominal cavity was a total absence of signal, while treaties were observed, most apoptotic cells located in the retina and into the lower abdominal cavity. Following the observations described it was considered appropriate to make a count of apoptotic cells in various anatomical regions of control embryos and treated with OTA (10µg / L) and 24hpf 144hpf, to highlight and quantify the areas most affected by treatment with OTA. Embryos treated 24hpf showed a decrease of 10% of apoptotic cells. At 144hpf the number of apoptotic cells increases significantly at the tail of the central nervous system, and particularly from the trunk and urogenital apparatus, in samples treated with ochratoxin A. In control samples at those areas was observed partial or total absence of positivity.

4. DISCUSSION AND CONCLUSIONS

Our research has shown that ochratoxin A is highly teratogenic because it causes morphological changes in embryos treated in the early stages of development, in addition to cyto-toxic in late development stages, it alters tissue homeostasis by increasing the number of cells in apoptosis. The results obtained by TUNEL are consistent with the findings of recent studies linking an increase in apoptosis of ochratoxin A by the ability to induce oxidative stress and lipid peroxidation. In conclusion, on a practical basis, who formulate fish feeds and feed fish must take into consideration the usage of OTA-contaminated aquatic feed and feed ingredients could represents a dangerous pollutant to the aquatic ecosystem.

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