SESSIONE 4

ASPETTI EMERGENTI IN ECOTOSSICOLOGIA

SESSIONE 4A: NANOECOTOSSICOLOGIA ACQUATICA

TiO_2 nanoparticles effects to saltwater organisms: preliminary results

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- **Abstract – The toxicity assessment in saltwater media is recognised to be harder than in freshwater ones mainly due to salinity and ionic strength. In this research, the *in vivo* effects of TiO_2 nanoparticles were checked considering a traditional exposure scenario on *Phaeodactylum tricornutum* and *Artemia franciscana*. Preliminary results evidenced an EC_{50} in the range 25–64 mg L^{-1} to *P. tricornutum*, while toxic effects were observed to *A. franciscana* after 24 h between 25 67 mg L^{-1}. Criticisms were identified about exposure scenarios and general testing conditions.**

Keywords:
(saltwater**;** toxicity test; TiO_2 nanoparticles)

1. INTRODUCTION

TiO_2 nanoparticles (nano-TiO_2) are employed in many industrial activities (Grzechulska-Damszel *et al.*, 2005; Fujishima *et al.*, 2008; Chen and Poon, 2009) as well as in common consumer products (Joner et al., 2009). Being generally assumed that nano-TiO_2 can enter the aquatic environment (Baun *et al.*, 2008), the assessment of nano-TiO_2 toxicity to aquatic organisms has become a priority. Although some papers (Figure 1) are already available about its toxicity to various freshwater species, only few studies deal with saltwater ones, and rarely using *in vivo* toxicity tests (Cattaneo *et al.*, 2009). Actually, salinity and ionic strength of both natural and artificial saltwater could limit the stability of nano-TiO_2 dispersions, affecting the size of their aggregates and, potentially, speeding up their sedimentation processes. As a result nano-TiO_2 bioavailability is continuously changed (French *et al.*, 2009), making the analysis and interpretation of results really hard.

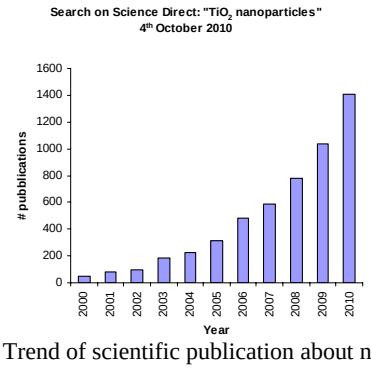

Figure 1 Trend of scientific publication about nano-TiO_2

In this work, the *in vivo* toxicity of nano-TiO_2 was evaluated via the growth inhibition test with the unicellular green alga *Phaeodactylum tricornutum* and the anostracan crustacean *Artemia franciscana* following the relative standardized protocols

2. MATERIALS AND METHODS

2.1 Materials

Aeroxide® P25 Titanium Dioxide powder was obtained from Evonik Degussa Corporation (Darmstadt, Germany). Ultrahigh-purity water, 18.2 MΩ cm resistivity (Millipore, Bedford, MA, USA) was used to prepare all solutions.

2.2 Physico-chemical characterization of nano-TiO_2 powder. Preparation and characterization of nano-TiO_2 dispersions in salt media

The nano-TiO_2 specific surface area was measured with the BET method (61 m^2 g^{-1}). Nano-TiO_2 morphology was analyzed with a Jeol JEM-3010 (Oxford Instruments, Tokyo, Japan) Transmission Electron Microscope (TEM). All nano-TiO_2 dispersions in culture media were prepared (step 1) by adding weighted amounts of nano-TiO_2 powder to a known volume of saltwater medium and (step 2) dispersing via probe sonication (UP-100H sonic probe - Hielscher, Teltow, Germany) for 10 min at 100 W in an iced bath. Size distributions of nano-TiO_2 suspended agglomerates were detected by means of a Nicomp DLS PSS 370 (Nicomp, Santa Barbara, USA) dynamic light scattering (DLS) instrument. The DLS analyses of nano-TiO_2 dispersions and media were performed 0, 24 and 48 h after the sonication process at 22 °C. Each DLS analysis consisted of five distinct runs of seven minutes each. Values are the average of at least three DLS analyses. The pH monitoring was carried on 0, 3, 6, 25 and 50 h after sonicating.

2.3 Organisms setting and test performing

The short-term chronic toxicity screening tests with *P. tricornutum* were performed according to UNI EN ISO 10253 (2006). The algal culture (lot. PT070509, SAG strain, Gent, Belgium) was kept at 20 ± 2 °C and 6000–10000 lx, obtaining a cellular density of more than 10^6 cells ml^{-1} after 3 ± 1 days. The test initial algal density was obtained by dilution of algal culture and ranged between 2×10^3 cells ml^{-1} and 10^4 cells ml^{-1}. *P. tricornutum* sub-populations were exposed to increasing concentrations of sonicated (10 min at 100 W) nano-TiO_2 for 72 ± 2 h at 20 ± 2 ◦C and 6000–10000 lx with a 14 h photoperiod in 3 ml 24 wells sterile polystyrene micro-plates with lids. Negative and positive controls were included in each experiment. Cell density was evaluated using a Bürker counting chamber. The tested (in

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triplicate) nominal concentrations were in the range 0.09 - 90 mg L^{-1}.
The acute immobilization bioassays (24 and 96 h) with *A. franciscana* were performed according to APAT and IRSA-CNR (2003). Prior to test, 100 mg of cysts (lot- AF/F2006, Gent, Belgium) were incubated in 12 mL of Instant Ocean® artificial seawater (35‰) at 25 °C (1 h under 100 W light and 23 h in darkness). After hatching for 24 h, Instar-I nauplii were collected and kept for an additional 24 h under the same conditions to reach the Instar-II stage. Nano-TiO_2 dispersions were tested in the range 0.1 – 1000 mg L^{-1}. About 10 nauplii were transferred in triplicate to each 3 mL well of polystyrene plates (24 wells with lids) containing 2 mL of sonicated (10 min at 100 W) nano-TiO_2 dispersion. A volume of 200 µL *P. tricornutum* culture were added only during the 96 h test after the first 48 h of exposure to feed the larvae. Negative and positive controls were included in each experiment. The well-plates were stored at controlled conditions (APAT and IRSA-CNR (2003) considering four scenarios (1) light and feeding, (2) no light, but feeding, to observe potential photocatalytic effects and (3) no feeding, but light, to verify effects on starvation, while (4) no feeding and no light is currently ongoing. Immobilization was evaluated every 24 h.

3. RESULTS

3.1 Characterization of nano-TiO_2 powder and nano-TiO_2 dispersions in saltwater media

Physico-chemical characterization of nano-TiO_2 powder evidenced a primary particle size between 15 and 60 nm (declared size: 21 nm), the crystallographic phases are anatase and rutile (EDX analysis); nanoparticles are mesoporous (bimodal distribution 2-4 nm; 10-90 nm). Nano-TiO_2 agglomerates surface charge was measured in NaOH solution at pH 8 (i.e. seawater) in order to determine the zeta-potential: the resulting value of -10.5 mV is characteristic of unstable dispersions. The addition of nano-TiO_2 powder to saltwater slightly altered the solution pH over time (8.10 ± 0.05). No difference was observed in the average hydrodynamic diameters of nano-TiO_2 agglomerates in both relative *P. tricornutum* and *A. franciscana* testing saltwaters. Mean diameters ranged between 200 - 900 nm immediately after the sonication process, while they increased over 1 µm after 24 and 48 h. These data highlighted the occurrence of an aggregation process, that could be qualitatively related to nano-TiO_2 dispersed powder sedimentation along time.

3.2 Exposure effects to *P. tricornutum*

The linear regression of a set of 42 data points from 8 toxicity tests suggested the following equation: $Y = 0.86X+11.78$ ($R^2 = 0.5646$, $\varepsilon = 19.51$). Nano-TiO_2 IC_{20} could be set at 10 mg L^{-1} (0-29 mg L^{-1}). Nano-TiO_2 IC_{50} could be set at 45 mg L^{-1} (25-64 mg L^{-1}). The lowest effect was detected at 0.9 mg L^{-1} and the maximum at 90 mg L^{-1}.

3.3 Exposure effects to *A. franciscana*

The different scenarios had been subdivided in 4 sub-scenarios, depending on the time: (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h.

The (1) scenario, “light and feeding”, evidenced an EC_{50} in a range between: (a) 25 and 67 mg L^{-1}; (b) 15 and 22 mg L^{-1}; (c) the minimum effect (3%) was detected at 2 mg L^{-1} and the maximum (100%) at 50 mg L^{-1}; (d) the minimum effect (3%) was detected at 4 mg L^{-1} and the maximum (100%) at 8 mg L^{-1}.
The (2) scenario, “no light, but feeding”, highlighted: (a) the minimum effect (3%) at 2 mg L^{-1} and the maximum (24%) at 8 mg L^{-1}; (b) the minimum effect (3%) at 8 mg L^{-1} and the maximum (28%) at 8 mg L^{-1}; (c) the minimum effect (3%) at 20 mg L^{-1} and the maximum (43%) at 16 mg L^{-1}; (d) the minimum effect (3%) at 16 mg L^{-1} and the maximum (47%) at 16 mg L^{-1}.
The (3) scenario, “no feeding, but light”, indicated: (a) the minimum effect (3%) at 0.5 mg L^{-1} and the maximum (91%) at 40 mg L^{-1}; (b) the minimum effect (3%) at 64 mg L^{-1} and the maximum (100%) at 20 mg L^{-1}; (c) the minimum effect (62%) at 8 mg L^{-1} and the maximum (100%) at 16 mg L^{-1}; (d) the effect was always 100% from 0.5 mg L^{-1} to 64 mg L^{-1}.

4. DISCUSSION

As a general observation, it was stated that the exposure concentration for *P. tricornutum* has been greater than that of *A. franciscana* even though the tested concentrations were nominally the same. Indeed, *P. tricornutum* population tends to drop down to the bottom of the testing wells lacking of agitating as it occurs to nano-TiO_2 that is almost completely deposited after 40 h (unpublished data). Thus the exposure to the algae is rapidly maximised. Conversely, *A. franciscana* that is a free swimming organism is exposed to the highest toxicant concentrations only for a limited period of time that is simply a part of the whole test duration. Therefore, it resulted that toxicity is decreasing with increasing exposure time because of a diminished toxicant availability.
The *P. tricornutum* EC_{50} showed to be comparable to data summarised in Kahru and Dubouguier (2010) review for algae: 65.5 mg L^{-1} (for nano-TiO_2, n=1) and 60 mg L^{-1} (for bulk metal oxides, n =4). The 24 h and 48 h *A. franciscana* (light and feeding) EC_{50} displayed a similar effects to that reported in the previously cited review for crustaceans being 67.7 mg L^{-1} (for nano-TiO_2, n =10), even if the bulk metal oxide presented an EC_{50} = 20 g L^{-1} (n =3). The 72 h and 96 h exposure times showed only a slight toxic effect.
The “no light, but feeding” *A. franciscana* scenario evidenced that the absence of light reduced the nano-TiO_2 toxicity, leading to the observation of very slight toxic effects in the 24-96 h exposure.
The “no feeding, but light” *A. franciscana* scenario highlighted that the association between starvation and the presence of light increased the toxic effects of nano-TiO_2 in the 24-96 h exposure.
About the algal bioassay, it must be signalled that for nano-TiO_2 concentrations greater than about 3 mg L^{-1} the algae population showed to be as unable to complete the cellular division appearing as aggregates reducing the reliability of growth inhibition reading procedure.
Are the commonly standard protocols exposure scenarios suitable for checking nano-TiO_2 toxic? Is it necessary to introduce some modifications/adaptations to improve the representativeness of the interaction between the model organism and the potential toxicant? Agitating or not agitating to facilitate the homogeneity of nano-TiO_2 dispersions? Does it make a non-sense to increase the level of stress to the target organisms in the considered scenarios? Such as an example, Hund-Rinke and Simon (2006) proposed to force the nano-TiO_2 exposure to algae in presence of UV-light that does not represent a real ecological situation.

How could it generate toxicity? Can it damage the cellular membranes or can it enter the cells?
Finally, the problem of quantifications should be discussed. Which metric may be used?

5. CONCLUSIONS

Of course, further studies are necessary within saltwater media and the relative model organisms, but the present preliminary results evidenced a certain level of data comparability with previous published researches even if about freshwater species.
A lot of questions raised about which is the best exposure scenario and if traditional ones are sufficiently reliable even in this case. Particular attention must be given to the analytical quantification of nano-TiO_2 dispersions and especially about its stability along time, that means during the performance of toxicity bioassays.

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Genotoxic potential of TiO_2 nanoparticles on isolated bottlenose dolphin *Tursiops truncatus*) leukocytes

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Abstract - Due to its extensive use, titanium dioxide nanoparticles (TiO_2NPs) are expected to enter the marine environment as an "emerging pollutant" in the near future. However, scarce information is still available about its impact on marine life. This research was aimed to assess the genotoxic potential of TiO_2 on bottle-nose dolphin leukocytes in comparison with human cells. Isolated leukocytes were exposed to TiO_2NPs (20, 50, 100 µg/ml) for 4, 24 and 48 h. Genotoxicity was detected by the Comet assay. Results showed that both the crystalline forms of TiO_2 (rutile and anatase) were genotoxic for dolphin leukocytes after exposure to 50 and 100 µg/ml for 24 and 48 h. Dolphin cells significantly more susceptible than human leukocytes to TiO2 particles.

Keywords: TiO2, marine mammals, genotoxicity.

1. INTRODUCTION

At the nanoscale, the properties of particulate matter differ peculiarly in term of conductivity and reactivity from those exhibited by the bulk form having the same chemical composition (Oberdörster et al., 2005). The novel physicochemical properties of nanoparticles NPs) is the rationale for their wide use in many industrial applications. On the other hand, the ability of NPs to enter the organism and interact with macromolecules raises concern for their potential effects on biological systems (Oberdörster et al., 2005). Titanium dioxide TiO_2 particles are used in many products/processes including cosmetics, sun screens, paints, pharmaceuticals, building materials, paper industry and waste water treatment (Aitken et al., 2006). Conflicting evidences about the toxicity of TiO_2 NPs have been reported; indeed, some investigations have found TiO_2 NPs being almost biologically inert for certain cultured mammalian cells (Linnainmaa et al., 1997); while other investigations demonstrated citotoxic and genotoxic effects of particulate TiO_2 (Falck et al., 2009). Moreover, epidemiological studies (Yamadori et al., 1986) and *in vivo* experimental exposures demonstrated that fine and ultrafine TiO_2 particles induce inflammatory processes, pulmonary damages, fibrosis and lung tumours (Bermudez et al., 2002). Aquatic ecotoxicology of TiO_2, and in general on NPs, is still scarce in spite that the release of "nano-wastes" is in the natural environments is expected to increase in the very near future (Moore, 2006; Owen and Depledge, 2005). At present, the limited literature on aquatic ecotoxicology of nanomaterials have dealt with the effects of NPs on invertebrates (Lovern and Klaper, 2006) and fish both *in vitro* and *in vivo*) (Federici et al., 2007; Vevers and Jha, 2008). No attention has already been paid to the potential effects of nanoparticles on marine top predators. In light of this lack of information, the main purpose of the present research was to investigate the susceptibility of toothed cetaceans, toward the two crystalline forms of TiO_2, anatase and rutile by a not invasive, *in vitro* approach. The bottlenose dolphin *Tursiops truncatus* was selected as study species being commonly reared in captivity. Human leukocytes were also investigated for comparing the susceptibility of these two species to TiO_2 exposure. The Alkaline Comet assay was used to test the DNA damage.

2. Material and Methods

Blood samples of *T. truncatus* were provided by the Adriatic Sea world "Oltremare" srl (Riccione, Italy); human ones were taken from two healthy male volunteers 24-30 years old. TiO_2 particles were purchased by Sigma-Aldrich; nominal particle dimensions were: rutile < 5000 nm; Anatase < 25 nm. Leukocytes were isolated by the lyses procedure. For in vitro exposure, TiO_2 particles were suspended in RPMI and sonicated for 30' at 35 kHz frequency. Three times of exposure (4, 24, 48h) and three doses (20, 50, 100 µg/ml) were tested. Hydrogen peroxide 100 µM) was used as positive control Genotoxicity was assessed by the Comet Assay, at pH ≥ 13 Singh et al., 1988) Cytotoxicity was detected using the Trypan Blue exclusion method. Multivariate Analysis of Variance (MANOVA) was used to analyze data.

3. Results

Transmission electron micrograph showed that anatase and rutile particles formed variously sized aggregates in RPMI solution after sonication. The size frequency distribution of the two suspensions (rutile and anatase) was similar, with a limited number of single particles < 100 nm and a large number of aggregates sized from a few to several micrometers. Cell viability was always very high, ranging between 70 and 100% after exposure to the highest doses (50, 100 µg/ml), for all the exposure times. The Comet assay data showed the absence of any dose-response trend after exposure of both dolphin and human cells. Anyway, a statistically significant increase of DNA fragmentation, with respect to controls, was observed in *T. truncatus* leukocytes after 24 and 48 h exposure to rutile at the highest doses. Rutile also induced DNA damage in human leukocytes exposed to 20 µg/ml. Anatase did not show any genotoxicity in human leukocytes, while a statistically significant effect was detected in dolphin cells exposed to 50 µg/ml (Fig1). The results of all the experiment are summarized in fig. 2, were the basal DNA fragmentation levels were subtracted by the ones induced by the TiO_2 treatment. These data are in agreement with the ones by Falck et al. (2009) which demonstrated that fine rutile is more toxic than nanosized anatase when tested on human bronchial epithelial cells.

4. Conclusion

Although preliminary, our findings suggest that bottlenose dolphin leukocytes are more susceptible toward TiO_2

particles exposure when compared with human cells exposed at the same range of doses and confirm the general opinion that cetaceans, especially odontocetes, are particularly subject to chemical stress induced by xenobiotic compounds (Fossi et al., 2007). Moreover, the results underline the importance of investigating the effects of emerging pollutants such as nanosized manufactured particles.

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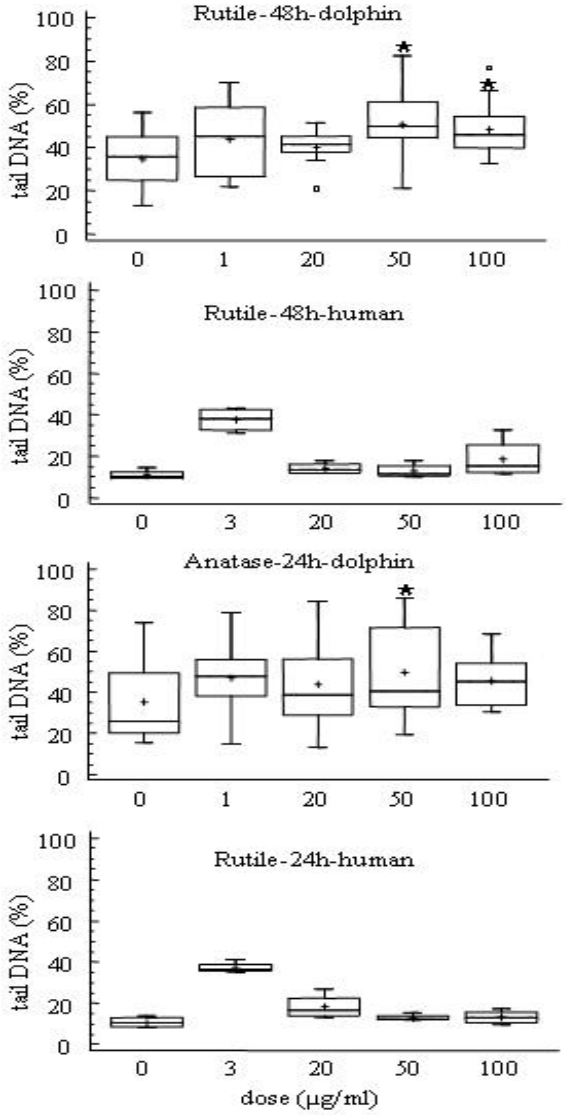

Fig. 1 Selection of results obtained after exposure of bottlenose and human leukocytes to TiO2 rutile and anatase on. * = $p < 0.05$

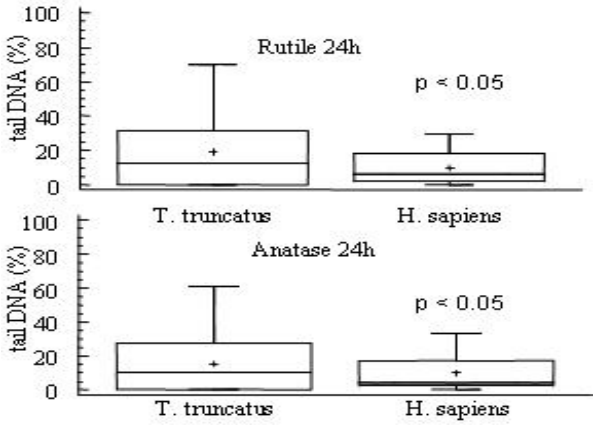

Fig. 1 Mean DNA damage after exposure to different doses of TiO_2 rutile and anatase. For a better comparison between the two study species, the basal DNA fragmentation levels were subtracted by the results of exposures.

Ecotoxicological effects of oxylipins from marine diatoms on benthic organisms

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Abstract – The effect of diatom-derived polyunsaturated aldehydes (PUAs) on development was tested in the sea urchin *Paracentrotus lividus*. PUAs blocked sea urchin cell cleavage in a dose dependent manner. Sub-lethal concentrations of decadienal delayed development of embryos and larvae which showed various degrees of malformations and increased proportion of apoptotic cells. Enzymes involved in PUA synthesis remain active for several minutes after cell-wounding and the PUA decadienal is detected in sea water for up to 14 days. Eggs and larvae from benthic organisms may come into contact with diatom PUAs in the field at the end of a bloom and local concentrations of PUAs may be high enough to potentially impact fertilization success and embryonic fitness of marine organisms.

Keywords: diatom aldehydes, apoptosis, sea urchin.

INTRODUCTION

Almost a decade ago, a group of short chain polyunsaturated aldehydes (PUAs) produced by diatoms cells were identified and shown to be responsible for a reduction in the reproductive success of copepods, the principal consumers of this group of microalgae (Miralto et al. 1999). It was demonstrated that the eggs produced by copepods that had been fed with mono-specific diatom diets, either failed to develop to hatching or hatched into malformed (i.e. teratogenic) nauplii that died soon after birth. These observations were also confirmed in the field, with low hatching success of copepod eggs during periods of intense diatom blooms (reviewed by Ianora and Miralto, 2010).
Such antiproliferative compounds may discourage herbivory by sabotaging future generations of grazers, allowing diatom blooms to persist when grazing pressure would normally have caused them to decline.
PUAs have important biological and biochemical properties, disrupting a number of critical stages in reproductive and developmental processes including gametogenesis, gamete functionality, fertilization, embryonic mitosis, and larval fitness and competence (reviewed by Caldwell, 2009).
Beside PUAs other oxygenated fatty acid derivatives such as hydroxides, oxo-acids, and epoxyalcohols (collectively termed oxylipins, including PUAs) were successively identified in diatoms (Fontana et al., 2007a). Oxylipins derive from fatty acid precursors through the action of enzymes activated within seconds after crushing of cells. These molecules function as wound-activated chemical defences, the production of which is triggered by cell damage during grazing or lysis of cells.
The enzymes involved in PUA synthesis have already been shown to remain active for at least 45 minutes after cell-wounding (Pohnert at al., 2002, Fontana et al., 2007b) resulting in high local concentrations of PUAs and other oxylipins that may have negative impacts on embryonic and larval fitness.
Of the oxylipins described so far, PUAs have been the most comprehensively studied because they are commercially available, inexpensive and sufficiently stable to allow for a range of laboratory bioassays. Here we review the effects of different diatom-derived PUAs, namely 2-*trans*,4-*trans*-decadienal,2-*trans*,4-*trans*-octadienal, 2-*trans*, 4-*trans*,7-octatrienal, 2-*trans*,4-*trans*-heptadienal, on early and later larval development in the sea urchin *Paracentrotus lividus*
Apoptosis induction in the pluteus stage of *P. lividus* after chronic exposure to decadienal is also reported. The deleterious teratogenic effects of the PUA decadienal on sea urchin embryos are compared with those on other marine invertebrates and copepods.

MATERIAL AND METHODS

Ecotoxicological test

Copepod fertilized eggs were freely spawned by female *Calanus helgolandicus* and incubated in 5-ml tissue-culture wells containing aldehydes (Sigma-Aldrich, Milan, Italy). The percentage of hatched naiplii was determined 48 h later when control untreated eggs had hatched. *Paracentrotus lividus* gametes were collected after injection of 0.2 mL of 0.2 M acetylcholine and fertilization was performed accordingly to Romano et al (2003). Soon after fertilization and envelope elevation, embryos were transferred to tissue culture wells containing aldehydes in 5 mL filtered sea water. Cleavage inhibition was assessed after circa 90 minutes from fertilization, when almost 100% of embryos were at the two blastomere stage and after 24 hours, the percentage of hatched larvae, as well as larval mortality was determined. PUAs were also tested for their teratogenic effect after long term incubation. After 48 hours of incubation with test compounds, the number of abnormalities and mortality were assessed according to Pagano et al. (1986).
Ciona intestialis oocytes were dissected from the oviduct and kept in normal seawater at 20°C until use. The chorion and follicle cells were removed by a 0.1% (w/v) trypsin treatment (Tosti at al., 2003). Spermatozoa were collected from the spermiduct and diluted in seawater immediately before insemination. Tadpole larvae were obtained from fertilized oocytes, kept at 18°C under slight rotation for 24 hours.

TUNEL labelling

C. helgolandicus embryos incubated in an aldehydic pool, were fixed in 4% paraformaldehyde for 2 hours at room temperature, rinsed several times in PBS and frozen in liquid nitrogen, at least three times, to fracture the carapace. Samples were then incubated for 12·h in 250·µL of 1·U·mL^{-}

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[1] chitinase enzyme (EC3.2.1.14; Sigma-Aldrich) in 50 mM citrate buffer, pH 6, at 25°C, to permeabilize the chitinous wall. After washing in PBS, embryos were incubated for 10 minutes in 0.1% Triton X-100 at room temperature, rinsed with PBS containing 1% BSA, and further incubated for 90 min in TUNEL solution at 37°C (terminal-deoxynucleotidyl-transferase-mediated dUTP Nick End Labelling; Roche Diagnostics GmbH, Mannheim, Germany). Pluteus *P. lividus* larvae and embryos were fixed for 1 h in 4% paraformaldehyde at room temperature. Fixed sea urchin embryos were washed several times in PBS, pH 7.4 then incubated for 1 h in 0.1% Triton X-100 and 0.1% sodium citrate, at 4°C. After washing in PBS containing 1% bovine albumin serum (BSA, Sigma-Aldrich) samples were incubated at 37°C for 90 min in TUNEL solution in a humidified chamber, in the dark. Control embryos were fixed and stained as described above.

RESULTS

Effect of PUAs on Sea Urchin development

Sea urchin eggs have been extensively used for ecotoxicological studies and to rapidly screen for bioactive compounds interfering with cell division processes. We have used this model also for studying the effect of PUAs such as 2-*trans*,4-*trans*-decadienal (decadienal), 2-*trans*,4-*trans*–octadienal (octadienal), 2-*trans*,4-*trans*,7-octatrienal (octatrienal), 2-*trans*,4-*trans*-heptadienal (heptadienal) on early and later developmental stages of *Paracentrotus lividus.* PUAs were found to block sea urchin cell cleavage in a dose dependent manner, but at different concentrations depending on the chain length of the molecules. Figure 1a reports the percentage of *P. lividus* embryos that were blocked after incubation with test compounds added to seawater at concentrations ranging from 0.658 to 32 µM. Percentage blockage of cell cleavage increased with increasing chain length from C7 to C10 PUAs, with arrest occurring at 27.27 µM with heptadienal, 16.13 µM with octadienal, 11.47 µM with octatrienal (which was slightly more active compared to octadienal), and 5.26 µM of decadienal. The saturated aldehyde tridecanal, also found in diatoms, did not interfere with first cleavage up to 25 µM. Higher concentrations were not tested due to solubility problems for this compound in sea water.

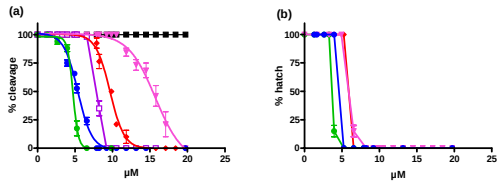

Figure 1. (a) Cleavage inhibition in sea urchin embryos following PUA treatment. (b) Percentage of hatched sea urchin larvae. Sea urchin embryos were treated at increasing concentrations of decadienal (green bullets), aldehyde mix (blue bullets), octadienal (red diamonds), octatrienal (blue open square), heptadienal (pink triangle) and tridecanal (black square). Values (means ± S.D.; N = 600) are the results of three different experiments (Romano et al. 2010).

All three PUAs exerted also a very strong dose-dependent effect on hatching success, with decadienal showing somewhat stronger effects than the other two aldehydes (Figure 1b). At concentrations of circa 3.0 µM, decadienal reduced hatching to <50%, but with octadienal and heptadienal, hatching viability was still >90% at this concentration. Total inhibition of hatching viability occurred at concentrations of 3.95 µM decadienal, 8.08 µM octadienal and 11.36 µM heptadienal, confirming that longer-chained aldehydes had somewhat stronger effects on hatching viability than shorter-chained aldehydes. No synergic effect on cleavage inhibition and only a slightly increased effect on egg hatching viability occurred when decadienal, octadienal and heptadienal were added as a mixture to the medium containing a 1:1:1 ratio of the three molecules.

When tested at sublethal concentrations, PUAs retarded development and induced malformations in sea urchin larvae. Incubation of newly fertilized eggs in decadienal at concentrations lower (1.32–5.26 µM) than those inducing cell blockage (6.58 µM) increased the number of abnormal sea urchin plutei and delayed the development of larvae or embryos which showed various degrees of malformations depending on the concentrations tested.

TUNEL staining

Plutei exposed to sublethal doses of decadienal were stained with the TUNEL fluorescent kit, specific for the detection of apoptosis. Plutei incubated in 1.32 µM decadienal showed somatic regions that were positively labeled, indicating occurrence of apoptosis in those tissues (Fig. 2c,d). The morphological aspect of the larvae compared to controls indicates a reduction in developmental rate and spicule elongation. At higher concentrations, the proportion of TUNEL-positive cells increases in a dose dependent manner. At 2.63 µM approximately 80% of larvae appeared to be positive in the entire body, presenting a more pronounced delay in the development of the arms (Fig. 2e,f). At 3.95 µM, embryos were quite similar to a late blastula and were apoptotic-positive throughout the entire body (Fig. 2g,h).

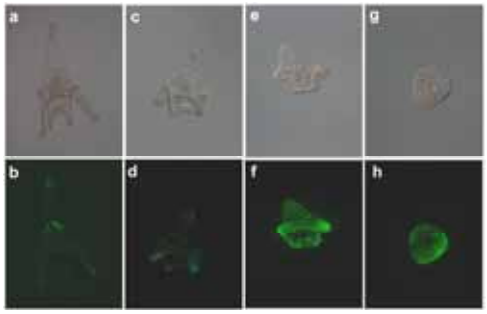

Figure 2. Tunel positive embryos incubated for 48 hours in decadienal at increasing concentrations (lower row) and corresponding images at transmitted light (upper row). Control Pluteus (a,b). Larvae after 48 hours incubation in decadienal 1.32 (c,d), 2.63 (e,f), 3.95 (g,h) µM (Romano et al 2010).

Effect of PUAs on *Ciona intestinalis* development

Decadieal negatively impact development also in *Ciona intestinalis.* The effect of this PUA was dose-dependent (Fig.3A), inducing a rapid decrease in the percentage of dividing eggs at concentrations >0.25 mg mL^{-1}, reaching 0% at a concentration of 0.8 mg mL^{-1}decadienal. Incubation at lower concentrations induced a dramatic

decrease in the percentage of larvae which did not develop beyond the blastula stage (Fig. 3B). Moreover, the percentage of abnormal larvae increased with increasing decadienal concentration. Developmental aberrations ranged from effects on sensory organs (otolith and ocellus), pigmentation, stunted elongation of the tail, and blockage before the 118-cell stage (gastrula) (Fig. 3B inset).

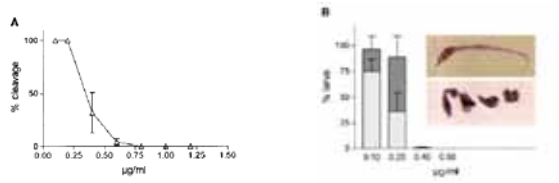

Figure 3. A: Percentage of first cleavage of *Ciona intestinalis* oocytes incubated 10 min at increasing concentrations of decadienal and then fertilized. B: Percentage of embryos reaching the larval stage after exposure to different concentrations of decadienal. Dark shading shows the percentage of abnormal larvae. Light shading shows the percentage of normal larvae. Bars indicate the standard deviation. Insert: top panel shows a normally developed *C. intestinalis* larva 24 hours after fertilization. Bottom panel: Various degrees of malformations such as stunted and elongated tail, lack of sensory organ pigmentation (Tosti et al. 2003).

Effect of PUAs on Copepods

Aldehyde pool purified from the diatom *Skeletonema marinoi* induced a decrease in the percentage of hatched nauplii with an increasing proportion of abnormalities at increasing PUA concentrations (Figure 4A). Nauplii coloured using TUNEL staining showed the presence of apoptotic tissue (Figure 4B).

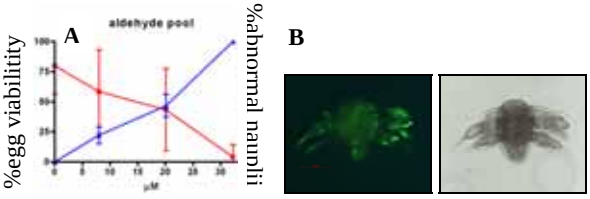

Figure 4. A) Effect of aldehyde pool from *Skeletonema marinoi* on egg viability and induction of abnormalities in *Calanus helgolandicus* nauplii. B) TUNEL staining on nauplius hatched from treated eggs at lower concentrations. Left panel: transmitted light image; right panel: fluorescent image of the same nauplius showing TUNEL-positive regions (Fontana et al., 2007).

DISCUSSION

Studies on the effect of PUAs on benthic organisms such as tunicates (Tosti et al., 2003), echinoderms and polychaetaes (Caldwell et al., 2009) indicate that their effect could be as severe as in planktonic animals such as copepods.

In the sea urchin *Paracentrotus lividus* decadienal completely blocked cell divisions when tested at high concentrations (≥5.26 µM). Hatching was blocked when a concentration of 3.95 µM was used. Other PUAs were slightly less active depending on the chain length. At sublethal decadienal concentrations embryos were able to survive, nevertheless presenting a certain degree of retarded development and presence of malformations.

In the tunicate *Ciona intestinalis*, sub-lethal concentrations of 0.2 µg mL^{-1} (1.32 µM) decadienal induced the formation of abnormal tadpole larvae which showed developmental aberrations ranging from effects on sensory organ pigmentation, reduction in elongation of the tail, and blockage before the gastrula stage, showing a sensitivity similar to that of *P. lividus.*

Caldwell and co-workers reported morphological measurements of *Psammechinus miliaris* echinopluteus larvae following sub-lethal exposure to decadienal (Caldwell et al., 2005). They found significant differences from controls after exposure to 0.1, 0.5 and 1 µg mL^{-1} decadienal concentrations (respectively 0.66, 3.29 and 6.58 µM), that are in the same range of decadienal concentrations reported to be active on *P. lividus* pluteus larvae. The same author reported that polychaetae larvae are very sensitive to aldehydes (Caldwell 2009). They found that decadienal induced morphological abnormalities in 9 day old larvae of *Nereis virens* exposed to this aldehyde during embryogenesis at concentrations of 0.01 and 0.05 µg mL^{-1} (0.066 and 0.329 µM respectively). Treated larvae showed abnormal development with incomplete ciliary band formation and the disruption of larval-body-plan.

Active decadienal concentration in the case of *N. virens* are thus lower than those required to induce abnormalities in sea urchins. These findings indicate differences in sensitivity to aldehyde exposure among benthic organisms. On the other hand, in pelagic copepods the concentration to induce malformations and apoptosis seems to be somewhat higher, 1 µg mL^{-1} (6.58 µM) (Ianora and Miralto 2010).

Previous studies conducted in our laboratory demonstrated that the PUA decadienal was able to induce apoptosis in early *P. lividus* embryos at concentration of 5 µg mL^{-1} (~33 µM), five times higher than the dose required to induce 100% cleavage inhibition (Romano et al., 2003). Chronical exposure of developing sea urchin embryos to decadienal can also induce apoptosis revealed at pluteus stage. At 3.95 µM decadienal, a strong effect was observed and almost all of the larvae were positively stained in the entire body.

Apoptosis has been shown to play two major roles during development, removing damaged cells throughout embryogenesis and sculpturing tissues during morphogenesis and metamorphosis (Zakeri et al., 2002) Apoptosis is also induced in cells exposed to various stress factors including toxicants, pollutants and heat shock. Several authors have in fact reported both physiological and induced apoptosis in sea urchin embryos (reviewed by Agnello and Roccheri, 2009).

Environmental exposure to PUAs may be dietary or by direct encounter with larvae or gametes, particularly if spawning coincides with diatom blooms. Senescent diatoms are known to undergo lysis and release lipoxygenase-end products (Vidoudez et al., 2008) that can generate a microenvironment in which gamete fertilization occurs, and and embryos and larvae develop and grow. It is thus reasonable to expect high local concentrations in the proximity of breakage of diatom cells. Ribalet (2007) estimated that such concentrations were well within the significant range for affecting growth and performance of surrounding organisms.

CONCLUSIONS

Our results indicate that even low concentrations of PUAs affect the developmental program in sea urchin embryos,

with evident malformations and apoptosis induction suggesting that most of these embryos are destined to die. Eggs and larvae of benthic organisms may come into contact with diatom PUAs in the field at the end of a bloom, with the mass sinking of diatoms to the sediment. Following diatom bloom termination the concentration of PUAs can be high enough to compromise the recruitment of future generations of these organisms. Understanding the fate of these compounds within the benthic realm could shed light on the role that they may play in ecosystem functioning.

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SESSIONE 4

ASPETTI EMERGENTI IN ECOTOSSICOLOGIA

SESSIONE 4B: ECOTOSSICOLOGIA DEI COMPOSTI NATURALI

Alterazioni biologiche indotte nei mitili, *Mytilus galloprovincialis*, esposti in ambiente naturale alla microalga bentonica *Ostreopsis ovata*

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Abstract - Negli ultimi anni, lungo le coste italiane, si è assistito ad un aumento degli eventi di bloom della microalga bentonica *Ostreopsis ovata*. Studi recenti hanno confermato la capacità di questa dinoflagellata di produrre biotossine, palitossina-simili, per le quali rimangono ancora poco conosciuti i meccanismi tossicologici e i rischi legati al trasferimento trofico. Lo scopo di questo lavoro è stato quello di indagare gli effetti biologici e tossicologici indotti nei mitili, *Mytilus galloprovincialis*, esposti alla microalga *O. ovata*, durante il bloom fitoplantonico verificatosi lungo la Riviera del Conero (An) nell'estate del 2009. I risultati hanno evidenziato una significativa inibizione dell'attività della pompa Na^+,K^+-ATPasi negli organismi esposti per i quali sono state anche osservate alterazioni del sistema immunitario e lisosomiale. I markers di stress ossidativo mostrano variazioni più limitate suggerendo come la tossicità indotta da *O. ovata* non sia mediata da una sovrapproduzione di specie reattive dell'ossigeno.

Keywords *Mytilus galloprovincialis, Ostreopsis ovata, biotossine algali, biomarkers.*

INTRODUZIONE

La presenza di microalghe platoniche potenzialmente tossiche nell'ambiente marino costiero è soggetta ad una continua attività di sorveglianza, attraverso appositi piani di monitoraggio da parte delle agenzie regionali per la protezione dell'ambiente (ARPA) che hanno permesso lo sviluppo di ricerche approfondite sulla produzione delle biotossine e sugli effetti tossici da queste indotte negli organismi marini e nell'uomo (Linee guida del Ministero della Salute 2007). Al contrario sono ancora scarse le conoscenze relative al problema delle fioriture di microalghe bentoniche tossiche. Tra queste la dinoflagellata bentonica *Ostreopsis ovata* è stata rinvenuta negli ultimi dieci anni in diversi tratti della costa italiana; eventi dannosi riconducibili a fioriture di *O. ovata*, riportati per la prima volta lungo le coste toscane nel 1998, sono stati registrati ad oggi in molte altre località costiere della penisola durante il periodo estivo, in alcuni casi sottoforma di consistenti fioriture e densi strati mucillaginosi che ricoprono il substrato biotico e abiotico (Totti et al., 2007).

L'impatto delle fioriture di *O. ovata* sull'ecosistema risulta estremamente importante tanto da incidere su settori economici quali pesca e turismo: si manifestano alterazioni della qualità e del colore dell'acqua, ipossia e/o anossia dei fondi e, ancora più grave, possono verificarsi morie di invertebrati planctonici e bentonici come molluschi, celenterati ed echinodermi (Rustighi e Casotti, 2005). Inoltre, i fenomeni di fioritura di *O. ovata*, che ormai da diversi anni richiamano in modo preoccupante l'attenzione degli amministratori e della comunità scientifica, hanno avuto risvolti allarmanti anche per la salute umana.

In Liguria nel 2005 circa 200 persone che avevano soggiornato in riva al mare o in zone adiacenti dovettero ricorrere alle cure ospedaliere a causa di una sintomatologia acuta a carico dell'apparato respiratorio a seguito dell'inalazione dell'aereosol marino contaminato (Icardi e Marensi, 2005).

Recenti studi hanno dimostrato che le biotossine di origine algale (incluse palitossine, saxitossine e yessotossine) possono causare numerose alterazioni fisiologiche, biochimiche e cellulari negli organismi marini. *Ostreopsis ovata* è in grado di produrre analoghi della palitossina, la maggior parte delle quali ascrivibili alla classe delle ovatossine, i cui effetti tossicologici rimangono ancora largamente sconosciuti (Ciminiello et al., 2010). E' noto come la palitossina rappresenti una delle sostanze naturali non proteiche più tossiche, con azione diretta sui canali di membrana, ed in particolare sulla pompa Na^+/K^+ ATPasi (Deeds e Schwartz, 2010). La potenziale pericolosità di queste molecole e le scarse conoscenze sui reali effetti tossici indotti dalle biotossine prodotte durante i bloom di *O. ovata* a carico degli organismi bentonici, hanno suggerito lo sviluppo di questo lavoro il cui scopo è stato quello di caratterizzare specifiche alterazioni biologiche (biomarker) nei mitili, *Mytilus galloprovincialis*, campionati nell'estate 2009, durante l'evento di bloom verificatosi lungo le coste delle Riviera del Conero (An, Italia). L'indagine sui biomarkers (che comprende marker di neurotossicità, marker di disturbo ossidativo, di alterazioni lisosomiali e di proliferazione perossisomiale) è stata integrata con i dati di tossicità acuta ottenuti con metodi tradizionali e sperimentali quali il mouse test (Mouse Biotossicological Assay, MBA) e il test di emolisi su eritrociti di montone (Haemolysis Neutralization Assay, HNA) adottati dagli Istituti Zooprofilattici Nazionali per il monitoraggio dei rischi sanitari legati al consumo di prodotti ittici contaminati da biotossine algali. In ultima analisi questo studio si propone di contribuire alla gestione del rischio legato alle fioriture di *O. ovata*, attraverso un approccio innovativo che prevede l'utilizzo dei markers biologici, a supporto dei test convenzionali, allo scopo di comprendere il reale impatto tossicologico delle biotossine prodotte da questa microalga bentonica sugli organismi marini esposti, anche in riferimento ai risvolti sanitari a carico della salute pubblica osservati negli ultimi anni.

MATERIALI E METODI

L'area di studio scelta è quella del promontorio del Conero, dove *O. ovata* è comparsa fin dall'estate del 2006; i siti di raccolta si riferiscono a quello di Ancona Nord (AN) e di Cooperativa Portonovo (CP) localizzati rispettivamente a circa 100 e 1000 m dalla riva e quindi diversamente influenzati dalla presenza della microalga che si sviluppa principalmente nelle immediate vicinanze delle coste.

L'area di Ancona Nord comprende il sito del Passetto in cui nel 2009 si è verificata una fioritura molto intensa di *O. ovata* accompagnata da casi di intossicazione per inalazione di aerosol marino a carico dei bagnanti che per quattro soggetti si è conclusa con il ricorso alle cure ospedaliere. I campionamenti sono stati effettuati nel mese di luglio, agosto e settembre 2009 comprendendo una finestra temporale che include periodi di totale assenza (luglio), di bloom (agosto) e di graduale scomparsa dell'alga nelle acque (settembre).

Subito dopo il prelievo l'intero tessuto molle di circa 30 organismi per sito sono stati dissezionati e immediatamente utilizzati per il saggio biotossicologico su topo (MBA) e il saggio di emolisi degli eritrociti (HNA), allo scopo di valutare la presenza di composti palitossina-simile nei mitili (Aligizaki et al., 2008). Il metodo biotossicologico ufficiale del Mouse Biological Assay per le DSP previsto dal Decreto 16 maggio 2002 del Ministero della Salute prevede la realizzazione di due protocolli sperimentali, da noi definiti Step 1 e Step 2, che prevedono l'inoculo su topo di due tipi diversi di estratti preparati a partire dai campioni di mitili, e precisamente:

-Step 1, prevede la realizzazione di un estratto in diclorometano in cui vengono concentrate biotossine con caratteristiche apolari (incluse acido okadaico, dinofisitossine, pectenotossine)

-Step 2, prevede la preparazione di un estratto metanolico che concentra le biotossine polari, e che rappresenta il metodo ufficiale per la determinazione di yessotossine. La comunità scientifica è ormai concorde nel sostenere che la positività allo Step 2 sia indice di presenza di altre biotossine interferenti come le palitossine o PSP.

Per l'analisi dei biomarkers, le ghiandole digestive, le branchie e l'emolinfa di circa 30 organismi per sito sono stati prelevati e opportunamente conservati fino al momento delle analisi. Tra i biomarkers di risposta specifica sono stati analizzati l'attività della pompa Na^+/K^+-ATPasi, la proliferazione perossisomiale, l'attività dell'acetilcolinesterasi. Poiché le palitossine sono coinvolte nell'alterazione delle pompe ATP dipendenti, con conseguente destabilizzazione delle membrane cellulari e sbilanciamento dell'equilibrio osmotico, è stata analizzata l'attività della pompa Na^+/K+-ATPasi; la proliferazione dei perossisomi, già indicata come biomarker di esposizione a una grande varietà di composti xenobiotici o di sostanze farmacologicamente attive, è stata misurata come attività dell'acil-CoA ossidasi, enzima associato specificatamente con questi organelli. L'attività enzimatica della acetilcolinesterasi, enzima implicato nella trasmissione dell'impulso nervoso, è stata valtata attraverso il metodo spettrofotometrico di Ellman. Tra i marker di disturbo ossidativo l'attività dei singoli sistemi antiossidanti (catalasi, glutatione reduttasi, glutatione S-transferasi, glutatione perossidasi e glutatione totale) è stata misurata attraverso specifici saggi spettrofotometrici, ed integrata con lo studio della capacità antiossidante totale di contrastare specifiche forma reattive dell'ossigeno come il radicacale perossilico e idrossilico, misurata con il saggio gas-cromatografico Tosc-assay (Gorbi et al., 2008). La stabilità delle membrane lisosomiali e il contenuto dei lipidi neutri sono stati valutati su sezioni criostatiche di ghiandola digestiva dei mitili (Gorbi et al., 2008). Infine il rapporto tra granuliociti/ialinociti è stato analizzato in microscopia ottica come marker di alterazione immunitaria nei mitili campionati.

RISULTATI

I risultati delle analisi biotossicologiche sui mitili campionati sono riportati in Tabella 1; un esito negativo al mouse test è stato rilevato nei mitili campionati in entrambi i siti di indagine, durante il mese di luglio 2009, periodo antecedente all'evento di bloom di *O. ovata*. La presenza di composti palitossina-simile è stata osservata a partire dal mese di agosto nei mitili campionati nel sito più vicino alla costa (AN), mentre nel mese di settembre entrambe i siti analizzati hanno rivelato positività al mouse test (Step 2) e quindi presenza di biotossine nei tessuti dei mitili campionati. La positività ottenuta al mouse test è stata confrontata con i risultati del test emolitico HNA (Tabella 1); i risultati hanno mostrato una risposta di inibizione alla oubaina in quei campioni positivi al mouse test, e hanno inoltre permesso di confermare la natura palitossina-simile delle biotossine prodotte da *O. ovata*.

TABELLA. 1 RISULTATI DEI SAGGI BIOTOSSICOLOGICI DEL MOUSE TEST (MBA) E DEL TEST EMOLITICO (HNA) SUI MITILI CAMPIONATI IN LUGLIO, AGOSTO E SETTEMBRE 2009, NEI SITI DI INDAGINE DI ANCONA NORD (AN) E COOP PORTONOVO (CP)

	Sito	MBA		HNA
		Step 1	Step 2	*Inibizione dell'oubaina*
Luglio 2009	Ancona Nord	– – –	– – –	No
	Coop Portonovo	– – –	– – –	No
Agosto 2009	Ancona Nord	– – –	+ + +	Yes
	Coop Portonovo	– – –	– – –	No
Settembre 2009	Ancona Nord	– – –	+ + +	Yes
	Coop Portonovo	– – –	+ + +	Yes

– – – : assenza di mortalità

+ + + : 3/3 topi moti

L'esposizione alle biotossine prodotte da *O. ovata* ha determinato una significativa inibizione della attività della pompa Na^+/K^+-ATPasi e una risposta tempo dipendente della acil CoA-ossidasi (AOX, marker di proliferazione perossisomiale), con una inibizione dell'attività che si osserva durante i primi giorni di accumulo delle biotossine nei tessuti dei mitili. L'attività dell'acetilcolinesterasi (AChE) appare inibita nei mitili di Ancona Nord esposti a *O. ovata* rispetto a quelli di Coop Portonovo, non contaminati dalle biotossine, durante il periodo di agosto 2009, con variazioni che risultano tuttavia non sempre statisticamente significative.

In accordo con i risultati già descritti in letteratura per altre biotossine algali come yessotossina e saxitossina (Malagoli et al., 2007), anche le biotossine prodotte da *O. ovata* inducono una destabilizzazione delle membrane lisosomiali e un incremento della percentuale dei granulociti nell'emolinfa, popolazione cellulare coinvolta in numerosi meccanismi di risposta immunitaria inclusa la fagocitosi.

L'analisi dell'accumulo dei lipidi neutri (ORO) nei lisosomi della ghiandola digestive dei mitili mostra una diminuzione di questi prodotti nei mitili esposti, compatibile con l'insorgenza di processi autodigestivi negli organismi esposti a biotossine algali.

Gli effetti dei composti palitossina simile sul sistema antiossidante dei mitili appare più limitato; una moderata riduzione della catalasi, della glutatione reduttasi (GR) e dei livelli di glutatione totale (GSH) è stata osservata nei mitili esposti (positivi) di AN campionati durante l'agosto 2009 rispetto a quelli di Coop Portonovo (negativi) dello stesso periodo. La capacità antiossidante totale non ha mostrato variazioni statisticamente significative in nessuno dei gruppi analizzati.

L'analisi descrittiva delle componenti principali (PCA) dei biomarkers condotta per gli organismi di entrambe i siti durante i tre periodi di campionamento ha prodotto due componenti che spiegano circa l'80% della varianza totale (Figura 1). La chiara separazione dei tre periodi di campionamento di luglio (1) agosto (2) e settembre 2009 (3) mostra in prima analisi che esiste una variabilità stagionale delle risposte analizzate ascrivibile sia alla comparsa del bloom di *O. ovata* durante il mese di agosto, che alle normali variazioni stagionali dei parametri biotici e abiotici che notoriamente influenzano le risposte biologiche degli organismi utilizzati come bioindicatori. L'analisi delle componenti principali permette inoltre di osservare una netta separazione tra i campioni di AN (+) e quelli di CP (-) prelevati nel mese di agosto. Tale separazione appare principalmente influenzata dai parametri del Fatt. 1 che variano in maniera significativa (principalmente attività Na^+/K^+-ATPasi, % granulociti, attività AOX e della acetilcolinesterasi) e che quindi possono essere considerati come quelli principalmente coinvolti nella tossicità indotta dai composti palitossina-simile prodotti dalla microalga bentonica *O. ovata* (Fig. 1).

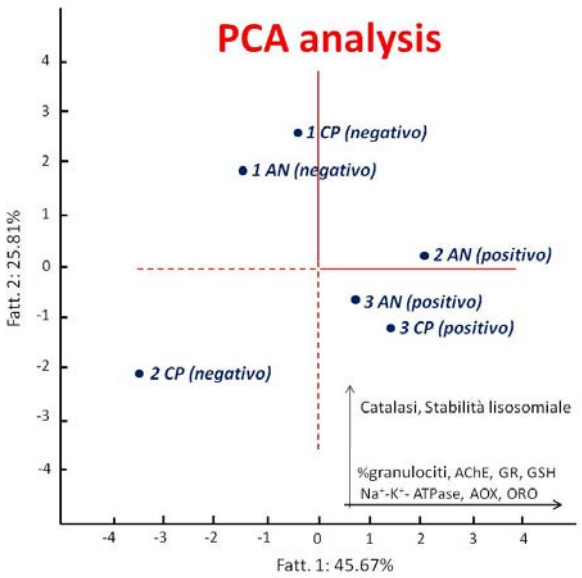

FIGURA. 1 ANALISI DELLE COMPONENTI PRINCIPALI (PCA) DEI BIOMARKERS ANALIZZATI NEI MITILI DI ANCONA NORD (AN) E COOP PORTONOVO (CP) PRELEVATI NEI TRE PERIODI DI CAMPIONAMENTO DI LUGLIO (1) AGOSTO (2) E SETTEMBRE 2009 (3).

CONCLUSIONI

In conclusione questo lavoro suggerisce la validità dell'analisi dei biomarkers per valutare i potenziali effetti biologici associati ai bloom di *O. ovata* e dimostra gli effetti di alterazione neurotossica, immunitaria e lisosomiale indotta dai composti palitossina-simili prodotti da questa microalga bentonica nei mitili dell'area della Riviera del Conero.

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STUDIO SULLA PRESENZA DI BIOTOSSINE ALGALI NELL'AEROSOL MARINO

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- **Abstract – Dinoflagellata *Ostreopsis ovata* (Fukuyo 1981) is a species can produce biotoxins extremely toxic. It is still to check the route of transmission of biotoxins of O. ovate man. hypothesis is the one most credited inhalation of marine aerosol. In this work were used, in an experimental, samplers automatic machines for marine bioaerosol with the consequent possibility to identify and quantify the algal toxins. The analytical results do not reveal significant levels of palitossina. through in vivo studies shows that inhalation of relatively low doses of palitossina is able to induce an marked pulmonary toxicity in experimental animals.**

Keywords: bioaerosol, *Ostreopsis ovata*, PJTX.

1. INTRODUZIONE

La microalga dinoflagellata *Ostreopsis ovata* (*Fukuyo 1981*), specie di origine tropicale ad habitus epi-bentonico, è stata segnalata lungo i litorali italiani, compresi quelli pugliesi, sin dal 2001. Le fioriture massive della specie sono state tipiche della stagione calda (*Fusetani et al 2009*), ed a queste, talvolta, sono state associate patologie riscontrate in bagnanti, pescatori e residenti nelle zone litorali (rinorrea, faringite, congiuntivite, dermatite, tosse, dispnea, febbre). Il fenomeno è stato evidenziato spesso dai mass-media in diverse zone del mediterraneo (*Villar-Gonzalez et al 2007*) e dell'Italia (*Ciminiello et al 2007*).
La problematica ha avuto dunque per la regione Puglia una valenza non solo ambientale, ma anche sanitaria, ed infatti nell'autunno 2008 è stato istituito un tavolo tecnico sull'argomento presso l'Assessorato Regionale alle Politiche della Salute.
Queste motivazioni hanno indotto l'ARPA Puglia ad adottare un piano di monitoraggio e sorveglianza avviato per la prima volta nella stagione estiva 2007 con lo scopo di rispondere in maniera pronta ed efficace alle esigenze ambientali e sanitarie del territorio pugliese.
Il monitoraggio condotto in questi due anni (*Poletti et al 2003*) ha permesso di identificare degli "*hot spot*" per le fioriture della microalga; in particolare l'area immediatamente a nord di Bari, tra gli insediamenti di Molfetta e S. Spirito (Fig. 1), è quella in cui si sono verificate le più alte concentrazioni (sino a 5.000.000 di cellule/litro in prossimità del fondo), tra i mesi di luglio e settembre.

Figura 1. Area *hot-spot*

Nell'ambito del programma di ricerca ISPRA-MATIM "*Ostreopsis ovata ed Ostreopsis spp.: nuovi rischi di tossicità microalgale nei mari italiani*", relativamente alle problematiche sia di tipo ambientale che sanitario legate alla presenza di fioriture di microalghe potenzialmente tossiche, è stata ravvisata l'opportunità di indagare gli aspetti inerenti lo sviluppo di aerosol marino ed i suoi possibili effetti tossici. Infatti, per quanto l'aerosol sia indicato come il principale veicolo di compromissione della salute pubblica anche nelle zone immediatamente circostanti le acque di balneazione, a tutt'oggi resta da dimostrare in modo inequivocabile la presenza della biotossicità e/o delle cellule (intere o in frammenti) di *O. ovata*.
Lo scopo di questa ricerca è stato, quindi, quello di fornire elementi certi su cui basare una metodologia scientificamente valida e condivisa a livello nazionale, da utilizzare nelle attività di controllo e tutela dell'ambiente e della salute umana. Lo studio è stato articolato in due distinte fasi: campionamento bioaerosol marino ed analisi delle biotossine algali con studio in vivo.

2. MATERIALI E METODI

2.1 Campionatori

Per il campionamento dell'aerosol marino l'ARPACAL si è avvalsa di due tipi di campionatori: un dispositivo di captazione per BioAerosol in vetro borosilicato tipo Biosampler (SKC), ed un campionatore per microinquinanti Air FlowPUF.
Il dispositivo Biosampler è un sistema con capacità di adsorbimento su liquido (soluzioni fisiologiche sterili apirogene, terreni di coltura selettivi, grasso siliconico) con movimento centrifugo e flusso di aspirazione di 12,5 litri/minuto, con possibilità di lavorare fino ad 8 ore senza il danneggiamento delle bio particelle adsorbite (Fig. 2).
Tale dispositivo è stato associato ad un campionatore portatile AirCube COM2, avente le seguenti caratteristiche : campo di regolazione del flusso 0,2-30 litri/minuto in un unico range dinamico senza ausilio di adattatori o orifizi, flusso massimo di impiego 28 litri/minuto, massima compensazione raggiungibile 2200 mm/H20, compensazione a controllo elettronico delle perdite di carico, attenuatore di pulsazioni con controllo di pressione incorporato.

La pompa aspirante, tarata con flusso di 10 litri/minuto, è dotata di regolazione automatica del flusso di aspirazione con valvola proporzionale motorizzata, flusso con dispositivo Mass Flow Meter e lettura istantanea ed integrata, impostazione del campionamento per tempi e volumi direttamente dalla tastiera, impostazione del flusso di campionamento da tastiera, rilevazione istantanea e calcolo della media per temperatura al contatore, temperatura ambiente, pressione barometrica atmosferica, flusso di aspirazione, velocità e direzione del vento. E' resa possibile anche la visualizzazione grafica dell'andamento del campionamento con calcolo della deviazione standard e la possibilità di controllo remoto mediante scheda GSM/GPRS.

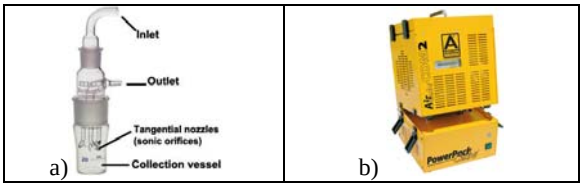


Figura 2. a) BioSampler b) Campionatore portatile AirCube.

Al sistema AirCube COM2 è stato associato un campionatore per microinquinanti Air FlowPUF. Quest'ultimo consente di campionare simultaneamente particolato solido sospeso e vapori organici ad un flusso di aspirazione compreso tra 120 e 600 litri per minuto. Il campionatore ad alto volume AirFlowPUF è completamente gestito da microprocessore ed attraverso il nuovo sistema di rilevazione flusso con tubo Venturi, in conformità ai requisiti della normativa ISO-5167, è in grado di gestire in modalità estremamente accurata tutti i campionamenti, anche per periodi prolungati (Fig. 3).
Uno speciale modulo di campionamento consente l'alloggiamento di due dispositivi d'adsorbimento, uno per particelle composto da una membrana filtrante in fibra di quarzo avente diametro 102mm e l'altro per composti organici costituito principalmente da una fiala avente 6 cm di diametro contenente della schiuma poliuretanica purificata (PUF).

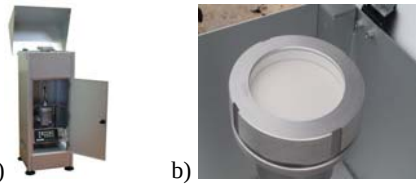


Figura 3. a) campionatore per microinquinanti Air FlowPUF b) membrana filtrante

2.2 Campionamenti
Nell'area di studio descritta i tecnici ARPACAL hanno eseguito quattro campionamenti di aerosol marino in quattro distinti momenti (18/09/09 – 02/10/09 – 20/10/09 - 19/11/09) ed in diverse condizioni meteorologiche (soleggiato – pioggia - vento forte – nebbia).
Entrambi i campionatori sono stati posizionati ad una distanza di circa 2-3 metri dalla battigia e ruotati occasionalmente in direzione del vento dominante al fine di garantire una captazione diretta dell'aerosol marino.
In concomitanza al campionamento dell'aerosol marino, i tecnici dell'ARPA Puglia hanno effettuato prelievi su colonna d'acqua e sul fondo al fine di poter stabilire le concentrazioni di *Ostreopsis ovata* nelle 4 giornate di campionamento (tabella 1).

Date di campionamento	Concentrazione di *Ostreopsis ovata* (cellule/litro)	
	Fondo	**Colonna**
18/09/09	5.792.000	23.520
02/10/09	3.685.000	25.600
20/10/09	20.500	240
19/11/09	6.700	120

Tabella 1. valori di concentrazione di *Ostreopsis ovata* (cellule/litro) su fondo e colonna d'acqua.

Il dispositivo in vetro borosilicato, la membrana filtrante in fibra di quarzo e la fiala contenente schiuma poliuretanica purificata (PUF) sono stati poi consegnati al Centro d'Eccellenza in Tossicologia Alimentare (CETA) dell'ARPACAL il quale ha proceduto all'esecuzione delle prove analitiche (acido domoico, acido okadaiko, yessotossina e palitossina); inoltre sono stati condotti studi *in vivo* durante i quali è stata somministrata la palitossina via aerosol ad animali da esperimento.

2.3 Preparazione dei campioni
In esito alle attività di campionamento si è proceduto alle determinazioni analitiche su:
-Membrana filtrante in fibra di quarzo da Air FlowPUF;
-Fiala contenente schiuma poliuretanica purificata (PUF) da Air FlowPUF;
-Liquido di gorgogliamento/campionamento da dispositivo in vetro borosilicato Biosampler.
Il solvente utilizzato nel processo di estrazione è stato il metanolo (*Ciminiello et al*,. 2006). La metodica ha richiesto:
-50 ml di MeOH per i filtri a membrana sottoposti poi a sonicazione (1 min per 3 volte);
-100 ml di MeOH per i PUF;
-10 ml di MeOH per il dispositivo in vetro borosilicato.
I campioni sono stati portati al volume di 2 ml, centrifugati a 14.500 rpm, filtrati e analizzati mediante LC-MS.

2.4 Cromatografia
Le prove analitiche sono state effettuate mediante cromatografo LXQ-IT – Thermofisher dotato di colonna del tipo Hypersil gold Thermmofisher (50mm x 2.1mm 1.9µm) termostatata a 30°C. La fase mobile per la determinazione dell'acido domoico, acido okadaico e della yessotossina è composta da H_2O e ACN addizionati di acido formico e formiato d'ammonio rispettivamente alla concentrazione di 50 mM e 2 mM. L'eluizione è stata condotta in gradiente (come riportato in figura 4), il flusso è stato pari a 200 µl/min.

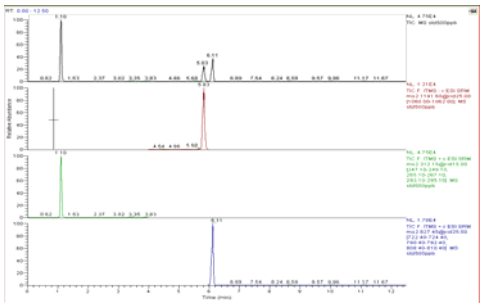


Figura 4. Cromatogramma di un multistandard (500ppb) di acido domoico, acido okadaico e yessotossina con estrapolazione delle transizioni relative alle tre molecole in esame.

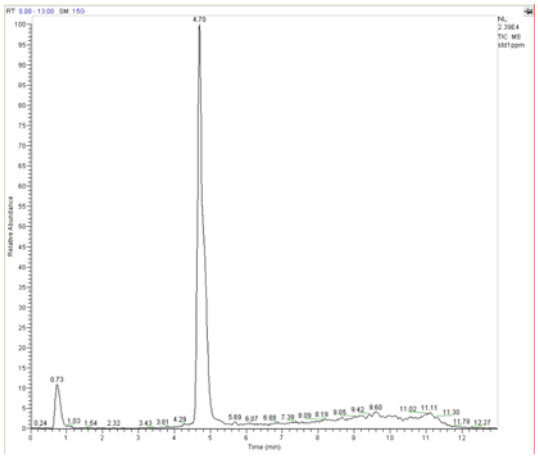


Figura 5. Cromatogramma di palitossina 1ppm

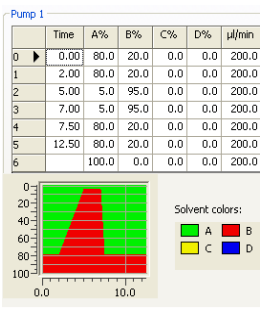
Per la determinazione della palitossina è stata usata una colonna Hypersil gold - Thermofisher (50mm x 2.1mm 1.9µm) termostatata a 30°C ed una fase mobile costituita da H2O e ACN addizionati di LiCl alla concentrazione di 100 ppb. L'eluizione è stata condotta in gradiente secondo quanto riportato in figura 6, il flusso utilizzato è di 200 µl/min.

2.5 Spettrometria di massa

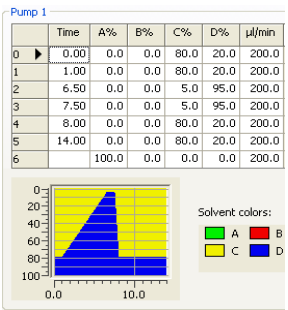
Gli esperimenti di spettrometria di massa sono stati effettuati mediante una trappola ionica lineare LXQ-IT ThermoFisher con sorgente ESI. La determinazione è stata condotta mediante metodica MS-MS, in positivo, per acido domoico, okadaico ed, in negativo, per la yessotossina. Sono state ricercate le seguenti transizioni:
Acido domoico *m/z* 312,5 $[M+H]^+$ *m/z* 248,10; 265,10; 294,10 (energia di collisione 15%; activation time 30ms);
Acido okadaiko *m/z* 827,45$[M+H]^+$ *m/z* 723,4 ; 791,4; 809,4(energia di collisione 25%; activation time 30ms);
Yessotossina *m/z* 1141,60 $[MH]^-$ *m/z* 1061,00 $[MH-SO_3]^-$ (energia di collisione 25%; activation time 20ms).
La palitossina è stata determinata monitorando i seguenti cluster ionici: *m/z* 906,5±4; 916,7±4; 1351,8±2; 1363,0±2 (SIM).

2.6 Validazione del metodo

Per la validazione del metodo sono state costruite le rette di calibrazione di seguito riportate e per ogni molecola è stato determinato il LOD. Sono state effettuate, inoltre, le prove di recupero a diversi livelli di concentrazione utilizzando e provando diverse miscele di estrazione.



Gradiente per AD, YTX, AO



Gradiente per PJTX

Il recupero è stato determinato a diversi livelli di concentrazione di palitossina: 50, 100 e 200 ppb. Le estrazioni eseguite secondo quanto riportato in letteratura (*Ciminiello et al*, 2006) hanno consentito un recupero pari al 60% circa. Ulteriori prove hanno dimostrato che la migliore miscela di estrazione è costituita da MeOH: H2O in rapporto 80:20. Il recupero effettuato mediante acetonitrile:acqua consente rese più alte ma anche maggiori interferenze nell'analisi cromatografica.

2.7 Studio *in vivo*

Lo studio è stato condotto su topi maschi CD1 del peso di 18-20 gr. mantenuti nelle stesse condizioni di temperatura (21°C), umidità (60±5%), ciclo buio-luce e con libero accesso al mangime e all'acqua, in accordo con le norme del Ministero della Salute in materia di stabulazione degli animali da laboratorio.
In accordo con le linee guida OECD per TGN°O 403 Acute inalation toxicity è stata utilizzata una camera in plexiglas a cui è stato collegato un sistema aerosol di tipo pneumatico.
Gli animali sono stati suddivisi in tre gruppi: al primo è stata somministrata via aerosol soluzione fisiologica (NaCl 0,9%); al secondo ed al terzo è stata somministrata palitossina rispettivamente nelle dosi di 450 e 900 ng/Kg. Per ogni singolo dosaggio sono stati posti 5 animali in camera di plexiglass, lasciati per 1 ora circa, e poi trattati per 30 min. con palitossina.
Durante il trattamento con 450 ng/Kg sono stati osservati: miosi; agitazione iniziale seguita da totale immobilità, tremori e posizione di difesa; a 6 ore dal trattamento immobilità e tremori diffusi.
Durante il trattamento con palitossina alla dose di 900 ng/Kg sono stati osservati: miosi; agitazione iniziale seguita da totale immobilità; tremori e posizione di difesa; ad 1 ora dal trattamento immobilità e tremori diffusi. Dopo 6 ore tremori diffusi. Dopo 12 ore sono stati osservati 2 decessi e tremori nei rimanenti animali. Dopo 18 ore si è verificata la morte dell'intera popolazione.
Durante il trattamento con NaCl 0,9% non sono stati osservati modificazioni comportamentali o alterazioni dello stato di benessere animale
Trascorse le 18 h tutti gli animali sono stati anestetizzati, sacrificati ed i polmoni espiantati, pesati e congelati rapidamente in azoto liquido.
Ad ogni grammo di campione è stato quindi aggiunto 1 ml di RIPA (Tris-HCl pH 7.4 1M; EDTA pH 8 0,5M; Triton X-100 ; NaF 1M, Na4P2O7 100mM; SDS 10%; DOC; Na3VO4 200mM e PMSF 100mM). L'omogenato è stato centrifugato per 30 minuti a 13.000 g ed è stata determinata la concentrazione della proteina nel surnatante (micro-BCA protein assay, Pierce Chemical CO., Roxiford, IL, USA).
Il lisato cellulare (30 µg/µl di proteina) è stato bollito per 5 minuti in tampone semplice e risolto attraverso SDS-P-PAGE 10%. Le proteine sono state poi elettroassorbite su filtri di nitrocellulosa (GE Healthacare) con pori da 0,45 µm. I filtri sono stati colorati con lo 0,2% di rosso Ponceau al fine di verificare il corretto caricamento e la corsa, lavati con TBST (20mM Tris-HCl a pH 8.0; 0.9% NaCl; 0.1% Tween 20), bloccati per 2 h con BSA (5%) in TBST (0,1%) ed incubati *over night* a 4°C con l' anticorpo specifico per COX-2 (1:500 in TBST all'1% di BSA, Cayman Chemical).
I filtri di nitrocellulosa sono stati lavati (4 volte x 15 min) con TBST ed incubati per 1 h con anti-rabbit IgG marcato con perossidasi (1:30.000 in TBST all'1% di BSA, GE Healthcare).
Infine, le bande sono state sviluppate usando un sistema di detezione della chemoluminescenza (ECL, Pierce).
Le stesse membrane sono state successivamente incubate per 2 h con l'anticorpo monoclonale specifico per la β-Actina (Sigma-Aldrich) utilizzato come "*housekeeping*

gene" e per 1 h con l'anticorpo anti-mouse IgG (1:30.000 in TBST all'1% di BSA) coniugato con perossidasi (GE Healthcare). Le bande sono state sviluppate utilizzando un sistema di detezione della chemoluminescenza (ECL, Pierce), e l'intensità della banda relativa alla proteina è stata analizzata attraverso l'MSF-300G Scanner.
Dopo aver sottoposto gli animali alle procedure sperimentali sopra citate, è stata eseguita la perfusione degli stessi mediante infusione di PBS e, successivamente, di formalina al 4% tramite il ventricolo sinistro al fine di lavare e fissare i tessuti. Quindi si è proceduto all'espianto del polmone. Il tessuto espiantato è stato disidratato immergendolo in una soluzione di saccarosio al 20% per 24h e successivamente tagliato al criostato in fettine di 20µm.
Le sezioni trasversali sono state disposte su vetrini polilisinati e colorati con il metodo ematossilina/eosina in modo da evidenziare i tipi cellulari. Le sezioni istologiche sono state trasformate in immagini digitali mediante telecamera (Leica DM 200) e software specifico; successivamente è stata effettuata l'analisi morfometrica mediante un sistema computerizzato di analisi di immagine.

3. RISULTATI

3.1 Analisi dei campioni di bioaerosol marino

Vengono di seguito riportati i risultati relativi alla determinazione analitica di acido domoico, okadaiko, yessotossina e palitossina nei quattro campionamenti:

Biotossina	Bioaerosol	Filtro	PUF
AD	< LRM	< LRM	< LRM
AO	< LRM	< LRM	< LRM
YTX –like	43,2 ppb	65,4 ppb	80 ppb
PTX	< LRM	< LRM	< LRM
LRM: Limite di rilevabilità del metodo			

Tab. 2. Campionamento del 18-09-2009

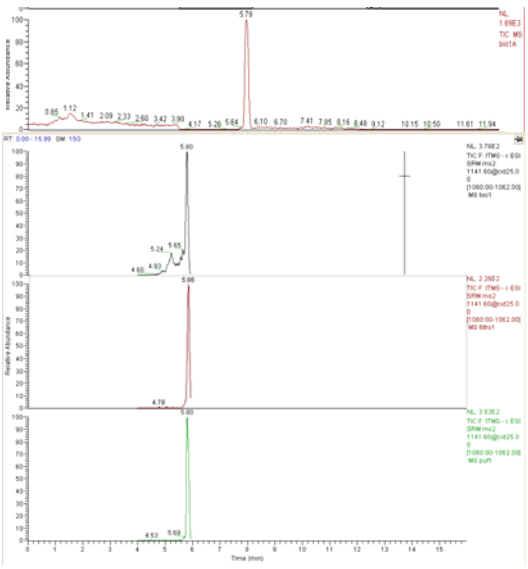


Figura6. Cromatogrammi relativi al campionamento del 18-09-09.

Come si evince dalla Tabella 9 l'unica biotossina determinata risulta una molecola yessotossina-like. Essa risulta strettamente correlata alla cooliatossina in quanto presenta le stesse transizioni di massa (*Holmes MJ et al, 1995*). La sua presenza è stata riscontrata nel solo campionamento effettuato in data 18.09.09 ed in tutti i sistemi di captazione del bioaerosol marino (membrana filtrante in fibra di quarzo da Air FlowPUF / fiala contenente schiuma poliuretanica purificata (PUF) / liquido di gorgogliamento/campionamento ottenuto da dispositivo in vetro borosilicato Biosampler).
Si ritiene di poter escludere che si tratti della yessotossina dal momento che la microalga elettiva produttrice della biotossina (*Prorocentrum lima*) è risultata assente nei campionamenti effettuati in colonna d'acqua e sul fondo.
Merita attenzione invece il riscontro qualitativo, anche se in modeste concentrazioni, sulla presenza di *Coolia monotis* nei campioni esaminati.

3.2 Risultati degli studi *in vivo*

La palitossina (450-900ng/kg) somministrata via aerosol ha provocato, nella maggior parte degli animali, significativi cambiamenti comportamentali e motori.
Gli esperimenti effettuati nel corso dello studio sperimentale, dopo trattamento di tossina al dosaggio più basso, hanno evidenziato negli animali trattati (10 animali per ciascun esperimento), miosi, spasmi muscolari cui ha fatto seguito la totale immobilità. Tali comportamenti sono stati osservati anche dopo 6 ore dalla somministrazione.
Il gruppo di animali trattati con la dose più alta della tossina presentavano già durante le prime fasi di osservazione miosi, tremori e posizione di difesa, convulsioni tonico-cloniche e totale immobilità (n=10). La mortalità, 12 ore dopo la somministrazione, è stata pari al 50% della popolazione. Gli animali sopravvissuti manifestavano un aumento dell'attività motoria e movimenti stereotipati. La mortalità, 24 ore dopo la somministrazione, è stata del 100%.
La somministrazione di un uguale volume di veicolo (NaCl 0,9%) non ha provocato alcuna modificazione comportamentale e sintomatologica in nessuno degli animali di controllo (n=10);
Negli animali sopravvissuti a 18 ore, si è proceduto ad una valutazione anatomo-patologica, effettuata dal CETA in Collaborazione con l'Università degli studi "Magna Graecia", che ha documentato, negli animali trattati ai diversi dosaggi una marcata iperemia a livello polmonare di tipo dose dipendente (vedi fig.9), mentre negli animali controllo non è stato evidenziato alcun danno polmonare. Inoltre, è stata osservata stasi ematica a livello epatico e cardiaco; da ciò si deduce che diverse popolazioni cellulari risultano sensibili alla pali tossina.

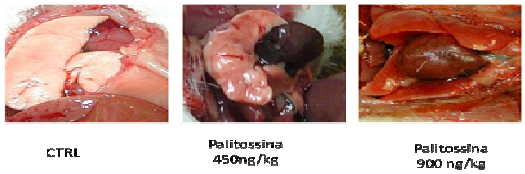


Figura 7. Analisi anatomo-patologica degli animali (controllo e trattamento con palitossina alla dose di 450 e 900ng/Kg).

Per confermare ulteriormente i nostri dati sperimentali, sui tessuti polmonari espiantati sono stati eseguiti studi di morfologia (colorazione eosina/ematossilina) che hanno

evidenziato a livello polmonare una marcata infiltrazione ematica dose dipendente rispetto al controllo.

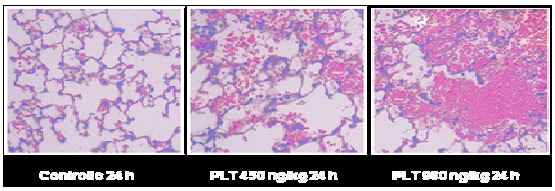


Figura 8. Esame istologico delle sezioni polmonari degli animali trattati con veicolo (A), palitossina alla dose di 450ng/kg (B), palitossina alla dose di 900ng/kg (C).

Al fine di valutare l'entità del processo infiammatorio sono stati eseguiti esperimenti di western-blotting per determinare quantitativamente la COX-2, proteina la cui espressione è indotta nel fenomeno infiammatorio. I dati ottenuti dimostrano come vi sia un significativo aumento dell'espressione della COX-2 a livello dei polmoni dei topi trattati con palitossina. In particolare le analisi mediante western-blotting, riportate in fig.11, documentano come la palitossina sia in grado di determinare un aumento dose dipendente dell'espressione di tale proteina.

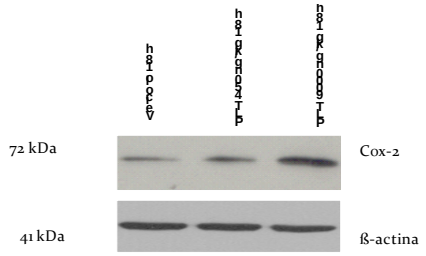


Figura 9. Analisi mediante western-blotting dell'espressione della COX-2.

4. DISCUSSIONE

I risultati dello studio consentono di poter concludere che:
Tra i due sistemi di campionamento utilizzati il dispositivo BioSampler in associazione con AirCube COM2 si è rivelato ottimale in quanto la matrice ottenuta oltre che risultare meno complessa ha richiesto volumi inferiori di solvente per l'estrazione (in pieno accordo con i principi della green chemistry).
L'unica biotossina determinata è stata una molecola yessotossina-like. La molecola potrebbe essere la cooliatossina, strettamente correlata alla yessotossina. La presenza di quest'ultima è stata esclusa in quanto non era presente *Prorocentrum lima*, la microalga responsabile della sua produzione, mentre è stata riscontrata la *Coolia monotis*, produttrice appunto di cooliatossina *(Holmes et al 1995*).
La molecola yessotossina-like è stata determinata in tutte e tre le tipologie di campioni derivanti dai due sistemi di campionamento ma solo relativamente al campione del 18-09-09, in cui è stata riscontrata la maggiore concentrazione di *Ostreopsis ovata* (circa 6.000.000 cell/L).
Gli studi *in vivo* hanno permesso di documentare in maniera evidente la tossicità a livello polmonare della palitossina somministrata via aerosol: in particolare, l'analisi anatomo-patologica effettuata sugli animali trattati ha mostrato una marcata iperemia a livello polmonare confermata nell'esame istologico da un'elevata infiltrazione ematica; l'analisi mediante western-blotting, ha rivelato come la palitossina sia stata in grado di provocare un aumento dose-dipendente dell'espressione della COX-2, marker di infiammazione.

Il mancato riscontro analitico di palitossina nei campioni analizzati non ci consente di escluderne la presenza; è ipotizzabile che, sperimentando l'uso di una strumentazione analitica differente (rivelatore di massa tipo orbitrap), si potrebbe aumentare di molto la sensibilità del metodo consentendo la determinazione di palitossina anche a concentrazioni molto basse.
Il gruppo di lavoro ritiene verosimile che durante il processo di campionamento le molecole possano danneggiarsi e deteriorarsi; tale considerazione suggerisce l'ipotesi di poter sperimentare un processo di campionamento ad intervalli regolari da cui ne derivino aliquote (nel caso del Biosampler) o cambi di filtro (per l'AirCom2) che riducano significativamente il possibile danneggiamento termico e/o ossidativo.
Di sicuro interesse potrebbero risultare futuri campionamenti in cui si verifichino congiuntamente condizioni di forte mareggiata, elevata formazione di bioaerosol e piena fioritura di microalghe tossiche. Ciò al fine di poter espletare ulteriori prove di conferma ed approfondimenti di indagine.

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SESSIONE 4

ASPETTI EMERGENTI IN ECOTOSSICOLOGIA

SESSIONE 4C: DESTINO AMBIENTALE ED EFFETTI DELLE SOSTANZE DI ORIGINE FARMACEUTICA E COSMETICA

"Green Chemistry" and ecotoxicity: the example of ionic liquids

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Abstract – Two ionic liquids were assessed for ecotoxicity and biodegradation. *N*-Alkyl-*N*-methylmorpholinium and *N*-alkyl substituted 1,4-diazabicyclo[2.2.2]octane (DABCO) based ionic liquids (ILs), *N*-alkyl-DABCO, bearing short alkyl chains are characterized by a low toxicity to *V. fischeri*, although toxicity significantly increases on increasing the alkyl chain length. Alkyl chain length affects also biodegradability in the 28 days tests; the higher level of biodegradation was found in both the series in the case of the ethyl (C2) derivatives. In the case of *N*-ethyl DABCO based IL, although biodegradability is still around 40%, and consequently this IL cannot be classified as "readily biodegradable", this value is similar to the more biodegradable functionalized imidazolium based ILs.

Keywords: ionic liquids; *Vibrio fischeri*; biodegradation

1. INTRODUCTION

The bioassays-based approach represents a fundamental point in the definition of ecological risk in the aquatic environment for promising chemicals as ionic liquids (ILs). In the registration process for all chemicals produced in or imported into the European Union above one metric tonne per year, the new regulatory framework for the Registration, Evaluation and Authorisation of Chemicals (REACH) adopted by the EU (2006) indicates the requirement of (eco)toxicological assessment. The Predicted No-Effect Concentration (PNEC) represents one of the major indicators required for the environmental hazard assessment of chemicals. PNEC consists of the identification of the environmental effects of chemicals on organisms and the determination of chemical concentration below which adverse effects in the environment (e.g., aquatic) are not expected to occur. The calculation starts from the available chemical and ecotoxicity data, derived from test organisms, such as bacteria, algae, crustaceans and fish in both acute and chronic toxicity experiments.

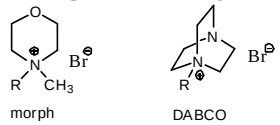
Over the past years, ILs have received considerable attention as potential green solvents or as materials for a wide range of applications in the area of green chemistry and engineering. This is prevalently due to their low vapour pressure (if any) and non-flammability that reduce air emission and improve operational safety with respect to the molecular solvents. However, the environmental risk assessment in terms of (eco)toxicity appears to be needful in order to evaluate the sustainability production of chemicals before any possible large-scale application. Recently, important data (both experimental and calculated) on the environmental impact of ILs have been published, showing that the ionic nature not necessarily confers an eco-friendly behaviour. Surely, the water solubility of many ILs may allow their widespread in the aquatic compartment and several ILs have been found to be toxic for different aquatic organisms, such as bacteria (Luis et al., 2010), unicellular algae (Kulaki and Lamberti, 2008), crustaceans (Samorì et al., 2007), molluscs (Bernot et al., 2005b) and fish (Pretti et al., 2006).

In principle this might have important consequences, such as, accidental spills and effluent discharges, in particular if their application in large-scale processes increases (e.g. accidental spills, effluent discharges). Fortunately, ILs not only have different degrees/levels of toxicity to aquatic organisms depending on species but toxicity is also strongly affected by IL structure. In this paper, we report experimental data on the aquatic toxicity and biodegradability of *N*-alkyl-*N*-methylmorpholinium (Morph) and *N*-alkyl substituted 1,4-diazabicyclo[2.2.2]octane (DABCO) based ILs as function of the alkyl chain length.

2. MATHERIALS AND METHODS

2.1 Ionic liquids

N-Alkyl-*N*-methylmorpholinium bromides were synthesized by addition to an acetonitrile solution of *N*-methylmorpholine (*ca*. 2M) an equimolar amount of the appropriate alkyl bromide and heating the resulting solution at 70 °C for 8-10 h, as previously reported (Bini et al., 2007). In the case of DABCO derivatives, to avoid the formation of the dialkylation product (Chiappe et al., 2009) an excess (10%) of DABCO was used. To a solution of DABCO in ethyl acetate (*ca*. 2 M) the appropriate alkyl bromide was added drop-wise and the resulting mixture was maintained at 50 °C for 12h. For all synthesized ILs, purity (> 99%) was checked by NMR (Bruker Advance II 250) and ESI-MS (LCQ Advantage spectrometer equipped with an ion-trap analyzer, ThermoElectron Company). All tested ILs are represented in Figure 1.



R = C_2H_5, C_4H_9, C_6H_{13}, C_8H_{17}, $C_{10}H_{21}$

Figure 1. Structure and alkyl chain length of methylalkylmorpholinium and alkyl-DABCO (N-alkyl substituted diazabicyclo[2.2.2]octane) based ILs.

2.1 CO_2 head space test

To evaluate the ultimate biodegradability of the test ILs, the "CO_2" Headspace test (ISO 14593, 1999) was applied. This method allows the evaluation of the ultimate aerobic

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biodegradability of an organic compound in an aqueous medium at a given concentration of microorganisms by analysis of inorganic carbon. The tested ILs, as the sole source of carbon and energy, was added at a concentration of about 20 mg/L of total organic carbon (TOC) to a mineral salt medium. These solutions were inoculated with activated sludge from a municipal wastewater treatment plant, washed and aerated prior to use and incubated in sealed vessels with a headspace of air. Biodegradation (mineralisation to CO_2) was determined by measuring the net increase in the TOC levels over time compared with not amended blanks. The test was ran for 28 days, sampling every 7 days, analyses were performed in three replicates. The extent of biodegradation was expressed as a percentage of the theoretical amount of inorganic carbon (ThIC) based on the amount of test compound added initially.

2.3 Microtox® bioassay (*V. fischeri*)

The Microtox® Test was performed according to the standard operating procedure using the Basic protocol (Azur Environmental, 1995), based on the ISO procedures (ISO 11348-3, 2007). The bacteria (*Vibrio fischeri*) were obtained from Ecotox LDS (Pregnana Milanese, MI, Italy) as freeze-lyophilized cells.

EC_{50} values, expressed as $\log_{10}$ (EC_{50}/μM) were reported as the means of three replicate determinations ± standard deviation.

3. RESULTS

3.1 *V. fischeri:* acute toxicity

The ecotoxicological test data are shown in fig. 2 (expressed as $\log_{10}$ EC_{50}/μM). DABCO- and morpholinium-based bromides, bearing the same alkyl side-chains as substituents on nitrogen, were characterized by similar EC_{50} values. Nevertheless, in both the series a strong correlation between toxicity and the alkyl side-chain length was found; a monotonic decrease in EC_{50} values for the bioluminescence activity of *V. fischeri* with increasing chain length of ILs has been observed. The lowest EC_{50} values were 1.3±0.1 for both DABCO C10 and *N*-methylmorpholinium C10, corresponding to an EC_{50} of ~20±4.5 expressed as μM. The highest EC_{50} values were 5.6±0.1 and 5.2±0.1 for DABCO C2 and *N*-methylmorpholinium C2, respectively. The corresponding values expressed as μM were 378.582 ± 71 and 161.90 ± 31 for DABCO C2 and *N*-methyl morpholinium C2, respectively. Parent compounds were also tested: the EC_{50} were 2.4 ± 0.4 and 3.8 ± 0.2 for DABCO and *N*-methylmorpholine, respectively.

3.2 Biodegradability

The biodegrability of morpholinium and DABCO-based bromides was assessed by the CO_2 headspace test. Concerning the sodium benzoate (reference substance) curve, a difference in the efficiency of biodegradation of about 10% was observed between filtered and not filtered samples, probably related to the occurrence of adsorption phenomena. Under the experimental conditions, the reference substance reached the maximum level of biodegradation (90% in not filtered samples) between day 7 and day 14 and all biodegradation rates of tested ILs were referred as percentage of reference, as shown in fig. 3 and 4. Two different experiments for evaluating the biodegradability rate of DABCO and morpholinium ILs were performed. All investigated ILs showed a low percentage of biodegradation (< 60%), with low differences between DABCO and morpholinium ILs and the alkyl chain length. Moreover, in both series the biodegradation trend showed that the higher percentage of biodegradation is reached between day 15 and day 21 after sampling. For DABCO ILs the percentage of biodegradation varied from 40% (C2, day 28) to 22% (C6, day 28). Morpholinium ILs showed a biodegradation efficiency lower than those of DABCO ILs: the percentage of biodegradation varied from 30% (C2, day 28) to 3-5% (C6, C8 and C10, day 28).

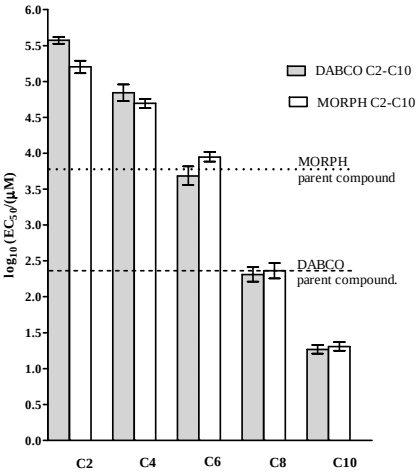

Figure 2. Acute toxicity (Vibrio fisheri) values (EC50) for DABCO-based and morpholinium ILs with different lateral chain length (C2-C10) after 30 minutes of incubation. Results are expressed as average log10(EC50/ M) ± standard deviation (n=3). Dot line represents the EC50 values of parent compounds (DABCO and N-methylmorpholine). Results are expressed as average log10(EC50/ M) ± standard deviation (n=3).
Significance, tested by one way ANOVA, Tukey's Multiple Comparison Test ($p<0.05$), evidenced that each DABCO-bar significantly differs from others DABCO-bars and that each morpholinium-bar significantly differs from others morpholinium-bars.

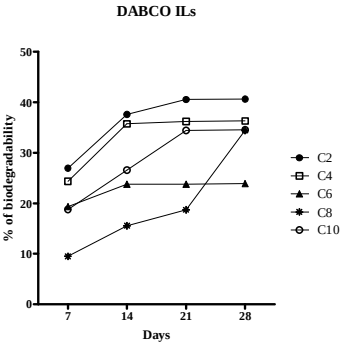

Figure 3. Percentage of biodegradability calculated for DABCO ILs with different lateral chain length (C2-C10). Observations were performed after 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data (n = 3).

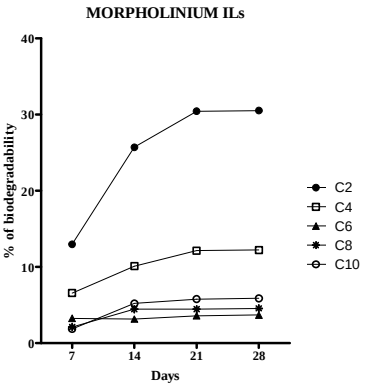

Figure 4. Percentage of biodegradability calculated for morpholinium ILs with different lateral chain length (C2-C10). Observations were performed after 7, 14, 21, and 28 days from the inoculums. Experiments were carried out in triplicate, levels were reported as average data (n = 3).

4. DISCUSSION

About the acute toxicity towards *V. fischeri*, these results represent the only source of data for DABCO-based ILs whereas for morpholinium-based ILs an EC_{50} value of 4.3 (expressed as $\log_{10} EC_{50}/\mu M$) was reported by Stolte et al. (2007) for *N*-methyl-*N*-butylmorpholinium bromide (Morph C4). This value is in agreement with our results giving an EC_{50} (expressed as $\log_{10} EC_{50}/\mu M$) of 4.7 for the same IL, although Stolte et al. (2007) used a different detection method (LUMIStox®).

On the basis of the data represented in fig. 2, it is evident that the transformation of both DABCO and *N*-methyl morpholine into the corresponding bromide salts bearing short alkyl chains reduces toxicity towards *V. fischeri* except to C8 and C10. The comparison of the EC_{50} values characterizing starting amines and the corresponding salts also shows that small amounts of the unreacted starting substrate (DABCO or N-Methylmorpholine) cannot significantly affect toxicity of the corresponding salts.

On the other hand, in agreement with the behaviour already evidenced with other ILs (imidazolium, pyridinium, ammonium, pyrrolidinium, piperidinium and so on), a correlation between toxicity to *V. fischeri* and lipophilicity can be envisaged: toxicity increases on increasing the alkyl chain length. However, when ILs bearing alkyl chains of comparable length are considered, morpholinium and DABCO-based ILs show toxicity towards *V. fischeri* significantly lower than imidazolium and pyridinium salts (Stolte et al., 2007).

The investigated DABCO and morpholinium based ILs did not show high levels of ultimate biodegradability: mineralization in 28 days was for all examined ILs lower than 60%; consequently, none could be defined as “readily biodegradable”.

5. CONCLUSION

The aim of this study was the investigation of the environmental impact of two classes of ILs based on aliphatic cyclic quaternarized amines, both bearing a further heteroatom (nitrogen or oxygen) on the cycle, on increasing alkyl chain length determining the effect on the marine bacteria *V. fischeri* and their biodegradability using the CO_2 head space test. All the tested salts had bromide as counteranion to reduce to the number of parameters that could affect the environmental impact evaluation. In conclusion, morpholinium and DABCO based ILs bearing short alkyl chains are characterized by a low toxicity to *V. fischeri*, although toxicity significantly increases on increasing the alkyl chain length. Alkyl chain length affects also biodegradability in the 28 days tests; the higher level of biodegradation was found in both the series in the case of the ethyl (C2) derivatives, *i.e.* the salts having the lower toxicity. In the case of *N*-ethyl DABCO based IL, although biodegradability is still around 40% and consequently this IL cannot be classified as “readily biodegradable”, this value is similar to the more biodegradable functionalized imidazolium based ILs.

ACKNOWLEDGEMENTS

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Simultaneous determination of pharmaceutical compounds in environmental samples

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Abstract - Pharmaceutical substances are synthetic compounds with very widespread usage relation to their therapeutic biological effects. These compounds and their bioactive metabolites can be continually introduced to the aquatic environment as complex mixtures via sewage treatment plants (STEP) (incomplete destruction), animal farms or leaching of landfills. In this work an analytical procedure involving solid-phase extraction (SPE) and gaschromatography–mass spectrometry (GC–MS) has been developed for determination of pharmaceutical compounds (caffeine, diclofenac, ketoprofen, ibuprofen and paracetamol) in a variety of aqueous samples (wastewater and surface water). Obtained results have demonstrated the suitability of the method at trace levels (ng l^{-1}) for multi-residue analysis of different types of water matrices.

Keywords: Pharmaceuticals; SPE; GC-MS;

1. INTRODUCTION

During the last three decades, the impact of chemical pollution has focused almost exclusively on the conventional "priority" pollutants, especially those acutely toxic/carcinogenic and industrial intermediates displaying persistence in the environment. This spectrum of chemicals, however, is only one piece of the larger puzzle of the risk assessment. Another diverse group of bioactive chemicals receiving comparatively little attention as potential environmental pollutants includes the pharmaceuticals. These compounds and their bioactive metabolites can be continually introduced to the aquatic environment as complex mixtures via sewage treatment plants (STEP) (incomplete destruction), animal farms or leaching of landfills (Daughton and Terners, 1999). They can represent a significant environmental risk if one considers on the one hand quantities potentially introduced into the aquatic environment and on the other hand the fact that they have been produced to be biologically active. STEPs have proved to be the main entry points of this contamination into the aquatic environment. Recent studies have documented the presence of a wide variety of pharmaceuticals in the environment worldwide, including antibiotics, anesthetics, anti-inflammatories, antitumor compounds, estrogens, lipid-reducing agents, diuretics, antidepressants, as well as illicit drugs (Castiglioni *et al.*, 2006; Khetan and Collins, 2007; Kummerer, 2001). The compounds analyzed in this study were diclofenac, paracetamol, ketoprofen, ibuprofen (Non-steroidal anti-infiammatory drugs), a group of the most commonly prescribed drugs (Jones *et al.*, 2002, Bottoni and Fidente, 2005), and caffeine (a stimulant). Because the pharmaceuticals are present usually in environmental waters samples at trace level, pre-concentration technique as Solid Phase Extraction (SPE) are necessary. Materials for SPE typically includes the use of octadecyl (C18) bonded silica cartridge (Togola and Budzinski, 2007), graphitized carbon black (Ding *et al.*, 1999), ethinylbenzene-divinylbenzene copolymer (Bolz *et al.*, 2000) and polystyrene-divinylbenzene (Weigel *et al.*, 2004). The selection of an appropriate solid phase is a difficult task, as the recoveries obtained for some compounds can be low. This problem is more evident in the simultaneous determination of several classes of pharmaceuticals. Following sample preconcentration, the analytical technique for the quantification of pharmaceuticals can be carried out with gaschromatography coupled to mass spectrometry, although, for acid compounds, it requires an additional step of derivatization (Rodriguez et al., 2003). The most commonly used derivatization reagents are: pentafluorobenzyl bromide (PFBBr) (Koutsouba *et al.*, 2003) and diazomethane (Ollers *et al.*, 2001) for carboxylic group and BSTFA (Boyd *et al.*, 2003), MTBSTFA (Rodriguez *et al.*, 2003) and *N*-methyl-*N*-(trimethylsylil) trifluoroacetamide (MSTFA) (Togola *et al.*, 2007, Quintana *et al.*, 2004) for hydroxyl and carboxyl functional group. The purpose of our study was to present a simple procedure for simultaneous determination of ibuprofen, paracetamol, caffeine, diclofenac and ketoprofen at trace level (ng l^{-1}) in environmental samples using SPE WCX preconcentration, followed by derivatization with MSTFA and GC-MSD analysis. In order to evaluate the reliability of the method, it has been applied to the analysis of surface waters and drinking waters.

2. MATERIALS AND METHOD

2.1 Chemicals and reagents

Acetone, ethyl acetate, methanol (HPLC reagent grade) and hydrochloric acid 37% (reagent grade) were purchased from J. T. Baker. Ultrapure water was obtained with a Milli-Q system (Purelab Option Q- ELGA). The silylation reagent dichlordimethylsilane (DCDMS), MSTFA (*N*-methyl-*N*-(trimethylsylil) trifluoroacetamide, purity >98%) and pharmaceutical products (ibuprofen, paracetamol, caffeine, diclofenac and ketoprofen) as well as pyrene and 1-hydroxypyrene, used as internal standards, were purchased from Sigma-Aldrich (purity >98%). Whatman GF/F filter was obtained from VWR International. Mecoprop (2-(4-chloro-2-methylphenoxy) propanoic acid) used as surrogate standard was obtained from Labor. Dr. Ehrenstoerfer.

2.3 Sample collection and solid-phase extraction

Sampling of different water samples was carried out in July 2010. The samples are: 1) surface water taken from a local river (Galeso River) in the first inlet of the Mar Piccolo basin of Taranto (Ionian Sea, Italy); 2) sewage/wastewater

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collector located in the second inlet of the Mar Piccolo; 3) surface waters sampled from Mar Grande of Taranto (Ionian Sea, Italy) influenced by urbanization; 4) drinking water. All glassware was silanized with DCDMS [10% (v/v)] in toluene in order to minimize the adsorption of trace level target compounds on the glass walls. First the glassware was rinsed with the silylation reagent, cleaned three times with toluene followed by three times with acetone, and then heated to 150 ◦C for at least 12 h. Samples have been collected in amber glass bottles, filtered on GF/F filter, acidified to pH 2 with HCL (3.5 M) to enhance trapping of the acid compounds on the SPE and extracted as soon as possible i.e. within 24h. 3 mL Solid-Phase Extraction cartridges packed with 60 mg of WCX sorbent, a polymeric phase with a carboxylic acid functionality (Agilent Technology), were used. SPE was performed under vacuum using 12-fold vacuum extraction box (Supelco) at a flow rate of 12- 15 ml min^{-1}. Before extraction loading SPE cartridge were conditioned with 3 ml of ethyl acetate and 3 ml of Milli-Q at pH 2. Sample extraction volume was generally 1000 ml. After the enrichment phase, the cartridge was dried for 1 h under vacuum. The analytes were eluted with 3 ml of ethyl acetate, 3 ml of ethyl acetate/acetone (1:1) and 3 ml of ethyl acetate/acetone/ammonium hydroxide (48:48:4, v/v/v) respectively. The elutes were collected in a silanized glass vial: the volume was reduced under a gently stream of nitrogen to 100 µl of ethyl acetate. For derivatization step 30 µl of MSTFA were added to the sample and reaction was carried out at 65°C for 35 min.

2.4 GC-MS analysis

Analyses were carried out using an gaschromatograph (7890 Agilent Technology) with PTV injector coupled to mass detector (5975C Agilent Technology). The mass spectrometer was used in electronic impact (EI) mode (70 eV electron energy) with ion source, quadrupole and transfer line temperatures of 230 °C, 150 °C and 280 °C, respectively. Injection of a 5 µl of sample was performed using the PTV injector in solvent mode and with the following temperature program: 50°C (0.5 min) to 250°C at 600°C/min (10 min) while vent flow was adjusted to 100 ml/min. The carrier gas was ultrapure Helium set a constant flow mode (1.5 ml/min). The chromatographic column was a PTE-5 (Supelco Inc. Bellefonte), 30 m × 0.32 mm ID × 0.25 µm film thickness. The GC oven was programmed as follows: 50°C (2 min), set at 10°C/min to 250°C (5 min) and 20°C/min to 280°C (2 min). Mecoprop were added to the sample, at the beginning of the extraction procedure, for recovery calculation while pyrene and 1-hydroxypyrene were used as internal standard for neutral and acid compounds respectively and added to the sample prior to derivatization step. For calibration curve 10 mg of each compounds was dissolved in 10 ml of methanol to give a 1000 mg l^{-1} stock solution. This was successively diluted to a mixed 10 mg l^{-1} solution in ethyl acetate containing all five compounds. From this, a series of mixed working standards solutions were prepared in the range of 1-50 µg l^{-1}.

3. RESULTS AND DISCUSSION

3.1 Optimization of the derivatization conditions

Target compounds as diclofenac, paracetamol, ketoprofen and ibuprofen contain hydroxyl and/or carboxyl groups and have therefore low polarity. Gaschromatographic separation of these compounds can be performed only after derivatization that convert functional groups into thermally stable, non polar groups. Silylation is the most widely used technique and MSTFA represent a typical reagent for derivatization of these pharmaceuticals compounds (Bound and Voulvoulis, 2006; Kosjek *et al.*, 2005; Togola *et al.*, 2007). It involves the replacement of an acid hydrogen with $SiMe_3$ to form tri-methyl (TMS) derivates. The reaction for TMS derivates occurs cleanly without artefacts; moreover since after derivatization no underivatised compounds were found when analysed by GC-MS, derivatization was considered complete. Derivatization reactions are affected by many possible factors as time, temperature, solvent and dose. With respect to reaction temperature and to the dosage of the derivatization reagent, 65°C and 30 µl of MSTFA was commonly used for reaction (Togola *et al.*, 2007). The reaction time for MSTFA were reported to vary (Jones *et al.*, 2003; Kosjek *et al.*, 2005). The variation might be caused by structural differences in the target compounds. In this study pharmaceuticals mixed solution were derivatized for different time (Fig. 1).

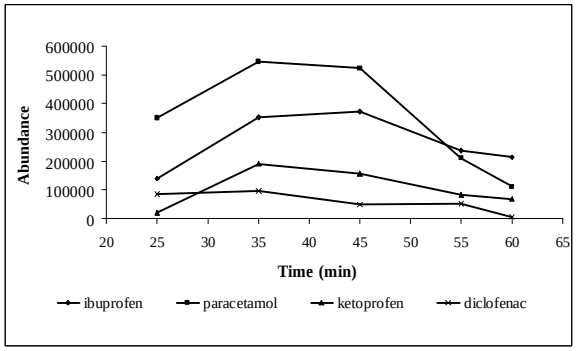

Figure 1. Mixed standards solution (100 µg/l of each compounds) of pharmaceutical compounds derivatized for different time.

Results showed that optimum time for derivatization was 35 min. Concerning solvent, toluene and ethyl acetate were tested in this study in order to determine the suitable medium of reaction. Results reported in Fig. 2 showed that ethyl acetate was to be most suitable solvent for MSTFA derivatization while when reaction was carried out without solvent (dry) derivatized products were very low.

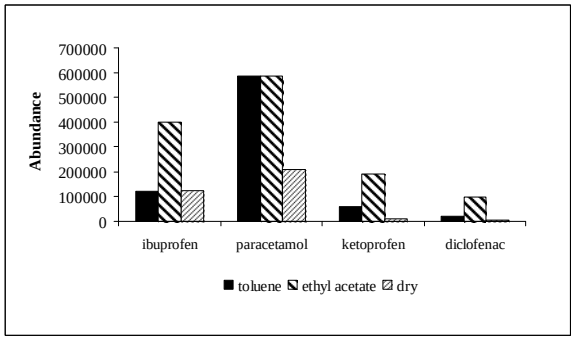

Figure 2. Mixed standards solution (100 µg/l of each compounds) of pharmaceutical compounds derivatized in different solvents.

3.2 Analytical development and sample analysis

A typical chromatogram of the TMS derivates of the selected pharmaceutical and internal standard compounds is showed in Fig. 3.

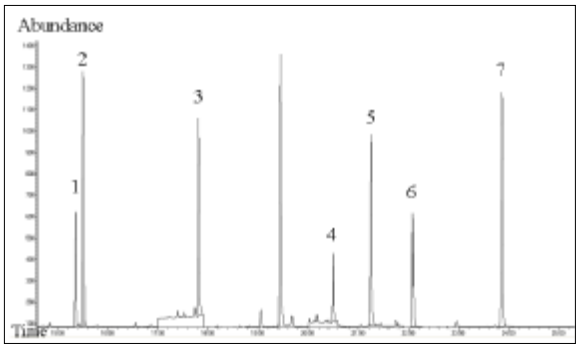

Figure. 3. Chromatogram in SIM mode of selected compounds after derivatization with MSTFA. (1. ibuprofen; 2. paracetamol; 3. caffeine; 4. pyrene; 5. ketoprofen; 6. diclofenac; 7. idroxypyrene.

The mass spectrum of each compound was characterized in full-scan mode and selected ion mode, was used for all quantitative measurements. Retentions time, m/z rations and limit of detections used for quantitative analysis was given in table 1.

Table 1. RETENTION TIME (T_R), MOLECULAR WEIGHT (MX), MOLECULAR WEIGHT OF TMS DERIVATES (MX-TMS) AND QUANTIFICATION ION OF PHARMACEUTICAL COMPOUNDS.

Analyte	Mx	Mx-TMS	Q. Ion (m/z)	Analytical LOD /pg injected
Ibuprofen	206	278	160	8.5
Paracetamol	151	233	214	2.5
Caffeine	194	-	194	12.5
Ketoprofen	254	326	282	17.5
Diclofenac	295	367	214	12.1

Linearity was tested with standard mixture in ethyl acetate at different concentration between 2.5 µg l^{-1} and 500 µg l^{-1}. Depending on the compounds correlation coefficient were 0.9916 for diclofenac and 0.9999 for caffeine. Relative standard deviation for replicate analysis ranged from 7 to 12 %. Recovery of pharmaceuticals ranged from 67 % for ketoprofen to 104 % for caffeine while RSD were between 5 and 9 %.

Method detection limit (MDL) was extrapolated from LODs taking into account the concentration factor from SPE procedure.

If a 100 % of extraction efficiency was presumed results of MDLs ranged from 0.05 for paracetamol to 0.35 ng l^{-1} for ketoprofen. These value enable use of this method to study the pharmaceutical products in natural waters that were found generally in range of ng l^{-1}. In most of the surface-water sampled in Mar Piccolo and Mar Grande of Taranto, levels of the pharmaceutical compounds were low ranging from n.d to 15.0 ng l^{-1} for caffeina, from n.d. to 10 ng l^{-1} for paracetamol while diclofenac, ketoprofen and ibuprofen were detected between n.d. and 3 ng l^{-1}. Higher concentration were found in rivers samples and in sewage/wastewater collector while no traces or lowest value of pharmaceuticals were found in drinking water and sea water. These results were in agreement with distribution of these compounds in other surface water (Togola *et al.*, 2007; Hilton et al., 2003; Zuccato *et al.*, 2000; Calamari *et al.*, 2003).

4. CONCLUSIONS

The method developed for analysis of aqueous samples containing the five pharmaceuticals selected using GC-MS detection is accurate, sensitive and reliable. The SPE WCX technique is an innovative phase for the simultaneous extraction of acid and neutral pharmaceutical compounds from environmental samples. This method has been shown to enable rapid and quick expensive. Further investigation to better characterized pharmaceutical compounds in environmental waters but also in biota and sediment are recommended. Actually, risk assessment does not indicate toxic risk especially for human exposure (Jones *et al.*, 2005, Webb *et al*., 2003). For aquatic organism exposure, research advances need to be more important before a real risk assessment if we consider long time exposure at environmental concentrations levels.

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