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Integrated analysis of the ecotoxicological and genotoxic effects of the antimicrobial peptide melittin on *Daphnia magna* and *Pseudokirchneriella subcapitata*

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ABSTRACT

Melittin is a major constituent of the bee venom of *Apis mellifera* with a broad spectrum of activities. Melittin therapeutical potential is subject to its toxicity and the assessment of ecotoxicity and genotoxicity is of particular interest for therapeutic use. Here we analyzed the biological effects of melittin on two aquatic species, which are representative of two different levels of the aquatic trophic chain: the invertebrate *Daphnia magna* and the unicellular microalgae *Pseudokirchneriella subcapitata*. The attention was focused on the determination of: i) ecotoxicity; ii) genotoxicity; iii) antigenotoxicity. Our main finding is that melittin is detrimental to *D. magna* reproduction and its sub-lethal concentrations create an accumulation dependent on exposition times and a negative effect on DNA. We also observed that melittin significantly delayed time to first eggs. Moreover, results showed that melittin exerted its toxic and genotoxic effects in both species, being a bit more aggressive towards *P. subcapitata*.

1. Introduction

Significant advances in modern medicine coupled to the indiscriminate use of potent and broad-spectrum antibiotics have generated high rates of microbial resistance and new challenges to human health (Arnold, 2007). This situation has renewed the interest in natural compounds which may contribute significantly to health in terms of prevention and treatment of many diseases. In this view, antimicrobial peptides (AMPs) have received an ever-growing interest as potentially powerful therapeutic tools in the fight against infectious diseases (Lazarev and Govorun, 2010). AMPs are defense molecules present and produced by all organisms, from bacteria to vertebrates and are effective against all microorganisms, including bacteria, fungi, viruses, and parasites. The majority of them accomplish their functions through a rapid disruption of cell membranes leading to cell death (Baek and Lee, 2010; Carballar-Lejarazu et al., 2008; Dobrzynska et al., 2005; Gopal et al., 2013; Haines et al., 2009; Vila-Farres et al., 2012).

Melittin is a small 26 amino acid residue peptide which is the main component of the Bee venom (BV) of *Apis mellifera* and has a significant haemolytic activity (Eisenberg et al., 1980). Its amino acid sequence is Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln and presents the typical features of AMPs with amphiphilicity due to the specific arrangement of amino acids in the chain (Eisenberg et al., 1980). In particular, nonpolar, hydrophobic and neutral amino acids are located at the N-terminal side whereas hydrophilic and basic amino acids are found at the C-terminus. Although it is soluble in water when in the form of monomer or tetramer, this polypeptide is incorporated rapidly into the membranes and disrupts the phospholipid bilayer. It has been hypothesized that melittin induces an increase in the activity of phospholipase A2, triggering countless effects on living cells. Moreover, it has been reported that melittin has multiple effects, including antibacterial, antiviral and anti-inflammatory activities in various cell types (Raghuraman and Chattopadhyay, 2007) and it is used in the treatment of several diseases such as arthritis, bursitis, tendonitis, herpes zoster, multiple sclerosis, wounds, gout, burns and

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infections (Chen and Lariviere, 2010; Lariviere and Melzack, 1996; Lee et al., 2007; Terwilliger and Eisenberg, 1982). Additionally, a great number of studies indicate that melittin inhibits the proliferation of cancer cells, and is involved in angiogenesis (Chen and Lariviere, 2010; Huh et al., 2010; Liu et al., 2002; Orsolic et al., 2003). Thus, melittin represents an attractive candidate for cancer chemotherapy (Hu et al., 2006; Moon et al., 2006, 2007; Putz et al., 2006; Son et al., 2007; Suh et al., 2006) because cancer cells are less likely to develop resistance against a membrane interacting peptide and the combination of a chemotherapeutic drug with melittin could be synergistic thereby reducing therapeutic doses of either one (Gajski and Garaj-Vrhovac, 2013; Hui et al., 2002). Although it is considered by some authors as a promising anticarcinogenic agent, it should be used with caution, since also at low concentrations it could induce genotoxic effect on human cells (Hoshina and Marin-Morales, 2014) and toxicity poses significant challenges to its therapeutic potential.

Concern about the presence and detection of toxic agents in ecosystems increased dramatically in recent years as a consequence of the fact that thousands of new substances are synthesized and released into the environment and it is impossible to avoid the exposure to them. While a large number of studies have investigated the ecotoxicity and genotoxicity of antibiotics, till now little has been done on the determination of the biological effects of AMPs. It is thus necessary to give indications of potential risks caused by medicinal products for the environment. In particular, the pharmaceutical research is facing the big challenge of antimicrobial resistance and new therapeutics need to be developed; in this scenario, AMPs represent an alternative that is being explored. At the moment, the use of AMPs is still at the beginning and before producing and using massive quantities of AMPs, it is necessary to evaluate their potential ecotoxicity.

For a long time aquatic contamination has been evaluated only by specific chemical analyses, but many years of experience have shown the inadequacy of such approach (Zhou et al., 2008). For the evaluation and monitoring of the water quality a series of methodologies, exploiting several bioindicators such as invertebrates, fish and algae, may be applied. The use of bioassay batteries allows to evaluate toxic effects at various biological levels. Among the tests available for the assessment of water quality and effluent acceptability, the ecotoxicity bioassay with *Daphnia magna* is one of the most widely used. This bioassay detects xenobiotics able to cause animal immobilization as a consequence of their toxicity. The current eco-toxicological requirements of the Directive 79/831/EEC for all new industrial chemicals involve the accomplishment of acute toxicity tests on fishes such as *Daphnia* (Sandbacka et al., 2000). In fact, chronic and acute tests with *D. magna* are among the most frequently performed studies in aquatic toxicology (Van den Brink et al., 2005). Moreover, several studies in *D. magna* showed that responses might change following different scenarios depending on the tested toxicant (Ohe et al., 2004). Previous findings also corroborated the sensitivity to various contaminants of *Pseudokirchneriella subcapitata*, located at the base of the food chain (de Paiva Magalhaes et al., 2014), and the need to use also this algae as a model for ecological risk. In particular, it is important to evaluate toxic effects, which can modify structure and productivity of the algae community and thus induce changes in the rest of the ecosystem (Martinez et al., 2014; Villem, 2011).

Moreover, to find emerging environmental contaminants, such as endocrine disruptors and genotoxicant, it is necessary to evaluate different biomarkers of the same indicator. DNA damage in aquatic organisms living in polluted environment could be considered as a biomarker for evaluating the genotoxic load and for eco-geno-toxicological risk assessment (Lee et al., 2009).

The present study was carried out in order to address the biological effects of melittin on two aquatic species, representative of two different levels of the aquatic trophic chain: the unicellular microalgae *P. subcapitata* and the invertebrate *D. magna*. Our analyses on these species might enable the coupling of the biological effects with possible ecological implications, which is fundamental before the development of AMPs as drugs or as components of biomaterial that could reduce the spread of bacterial infections. The attention was focused on the determination of i) ecotoxicity, in order to study how chemicals affect the environment and the organisms living in it; ii) genotoxicity in order to analyze the damages to the DNA; iii) the antigenotoxicity in order to verify DNA repair ability of the cells.

2. Materials and methods

2.1. Materials

Fmoc-protected amino acid derivatives, coupling reagents, and Rink amide p-methylbenzhydrylamine (MBHA) resin were purchased from Calbiochem-Novabiochem (Laufelfingen, Switzerland). Other chemicals were purchased from Sigma–Aldrich, Fluka (Buchs, Switzerland), or LabScan (Stillorgan, Ireland) and were used as received, unless otherwise stated.

2.2. Peptide synthesis

Melittin was synthesized using the standard solid-phase-9-fluorenylmethoxycarbonyl (Fmoc) method as previously reported (Cantisani et al., 2014). Briefly, the Rink amide MBHA resin (substitution 0.49 mmol g^{-1}) was used as the solid-phase support, and synthesis was performed on a scale of 100 μmol. Fmoc-protected amino acids (4 equiv relative to resin loading), were coupled according to the PyBop/HOBt/DIPEA method: Fmoc-amino acid (1 equiv), PyBOP (1 equiv), HOBt (0.5 mm in DMF, 1 equiv), and DIPEA (2 M in DMF, 2 equiv). The Fmoc protecting group was removed with 30% piperidine in DMF (v/v).

The crude peptide was purified by RP-HPLC on a LC8 Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV lambda-Max Model 481 detector using a Phenomenex (Torrance, CA) C18 column eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 20 to 80% over 20 min at a flow rate of 20 mL min^{-1}. Purity and identity were assessed by analytical LC-MS analyses by using Finnigan Surveyor MSQ single quadrupole electrospray ionization (Finnigan/Thermo Electron Corporation San Jose, CA), column: C18-Phenomenex eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B) from 20 to 80% over 10 min at a flow rate of 0.8 mL min^{-1}. The purified peptide (purity higher than 98%) was obtained with good yields (50–60%).

2.3. Test organisms and culture conditions

D. magna is a model herbivore in freshwater system, which links the aquatic food chains by growing on primary producers and being eaten by primary and further carnivores; therefore, is regarded as the most widely used aquatic indicator species. Daphnids were cultured in M4 medium (Elendt and Bias, 1990) at a constant temperature of 20 ± 1 °C, 16 h light and 8 h dark cycle photoperiod regime following the guideline OECD 211 (OECD, 2008). Culture medium was renewed three times a week and daphnids were fed with a suspension of green algae (Volker et al., 2013).

The green algae *P. subcapitata* was used because it is easy to culture and there are sufficient toxicity data available. It was maintained in laboratory cultures in a salt solution dissolved in ultrapure water (ISO, 2012). Culture was kept in Erlenmeyer flasks in the same conditions of *D. magna*. Exponential growing cells were

used for the bioassay.

2.4. Toxicity test on D. magna

2.4.1. Acute toxicity test

Initially, acute immobilization tests according to OECD 202 (OECD, 2004) of 48 h duration were conducted in order to assess the sensitive range of *D. magna* to different concentration of melittin. Briefly, neonates less than 24 h old were exposed to five different (50, 10, 5, 1, 0.1 μg/mL) concentrations of melittin and two control of non exposed. Each experiment was conducted using 20 daphnids divided into four replicates of five animals each. At the end of the test pH and oxygen concentration in the test-medium were measured. After 24 and 48 h, the immobilization of daphnids was recorded. They were considered immobile if they were not capable to swim after gentle agitation and the test were considered valid if the percentage of immobilization is less than 10% in the control. The EC_{50} and the EC_{10} were evaluated.

2.4.2. Chronic toxicity test

The experiments were performed according to the standard protocol for *D. magna* chronic toxicity testing OECD 211 (OECD, 2012). Briefly, daphnids less than 24 h old were used. They were exposed to a sub-lethal concentration (1 μg/mL) of toxic melittin and observed and fed daily for 21 days. Three replicates and one negative control were prepared, each consisting of 10 daphnids transferred to a new medium every 2 days. During that time, mortality, survival, time of the first brood, and newly born offspring were determined.

2.5. Toxicity test on P. subcapitata

2.5.1. Algae growth inhibition test

The growth inhibition test was assessed following a method of OECD 201 (OECD, 2006). Briefly, exponential growing algae cells with an initial concentration of 5×10^4 algae cells/mL was incubated in each well containing serial dilutions (starting from the concentration of 10 μg/mL with arithmetic progression of 1:2) of melittin on MBL medium. For each sample, five concentrations in a geometric series were tested in the concentration range first established in a preliminary test. Three replicates were performed per concentration. Plates were incubated in a light-temperature controlled chamber at 25 °C for 72 h with a photoperiod of 16 h:8 h light–dark. After 72 h a sample from each well was read in a spectrophotometer at 440 nm. The endpoint consisted of cell growth inhibition, which was measured after 72 h in a Burker cell coventing chamber and calculated by dividing the difference of the number of control and sample cells to the number of control cells.

2.6. Genotoxicity studies

All mobile and immobile daphnids from acute toxicity test were submitted to comet assay. In order to compare the genotoxicity of melittin between acute and chronic test and to evaluate the bioaccumulation damage, daphnids at 21 day and newly born daphnids were submitted to comet assay. Mobile daphnids exposed to 50 μg/mL were divided in two groups; one group was subjected directly to comet assay; while for the other group, the induction was stopped by suspending daphnids in a fresh medium and fed. After 24 and 48 h, daphnids were taken for comet assay to evaluate the DNA repair process. For evaluation of DNA damage in *P. subcapitata* cells, the treated cells and negative control (not treated) were collected by centrifugation and submitted to comet assay.

2.6.1. Comet assay to D. magna

The Comet assay to *D. magna* was performed according to the method described by Pellegri et al. (2014) and Buschini et al. (2004) with some modifications reported below. Treated organisms were suspended in 500 μL of PBS solution containing 20 mM (EDTA) and 10% (DMSO), and after they were subjected to mechanic homogenization. The resulting solution was filtered (Sigma Aldrich, 100 μm mesh) and transferred into an 1.5 mL Eppendorf tube. 40 μL of the solution was further gently re-suspended in 40 μL of Low Melting Point Agarose (LMA, 1%) and then transferred onto degreased microscope slides, previously dipped in Normal Melting Point Agarose (NMA, 1%). After solidification at 4 °C for 5 min, a second layer of 80 μL of LMA was added. Slides were placed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, pH 7.5) overnight at 4 °C to digest both the plasma and the nuclear membranes. Before electrophoresis, slides were incubated for 30 min in a freshly prepared alkaline buffer (300 mM NaOH, 1 mM Na_2EDTA, pH > 13). The slides were drained and placed in a horizontal electrophoresis tank in the same buffer for 30 min by applying an electric field of 25 V and adjusting the current to 300 mA. Finally, the slides were gently washed twice in a neutralization buffer (Tris–HCl 0.4 M, pH 7.5) for 5 min to remove alkali and detergent, and stained with 50 mL/mL DAPI (3 h).

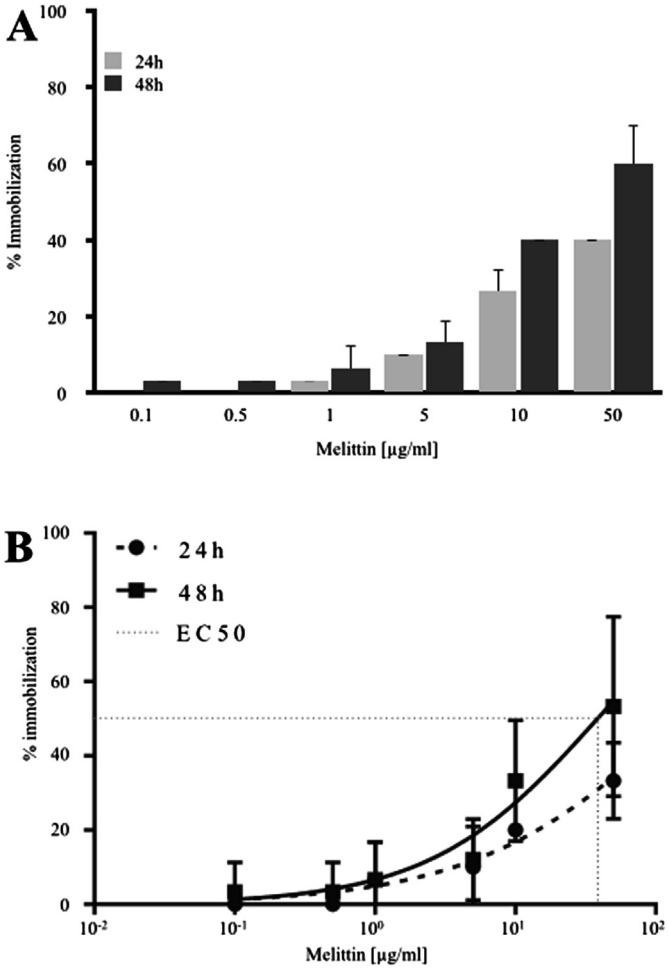

Fig. 1. Immobilization comparison of *D. magna* exposed to different concentration of melittin after 24 h and 48 h (A); Fitted curves by $\log_{10}$ at 24 h and 48 h (EC_{50} 38.49 μg with IC 95% of 22.29–66.45; EC_{20} 5.53 μg with IC 95% of 2.94–10.4) (B).

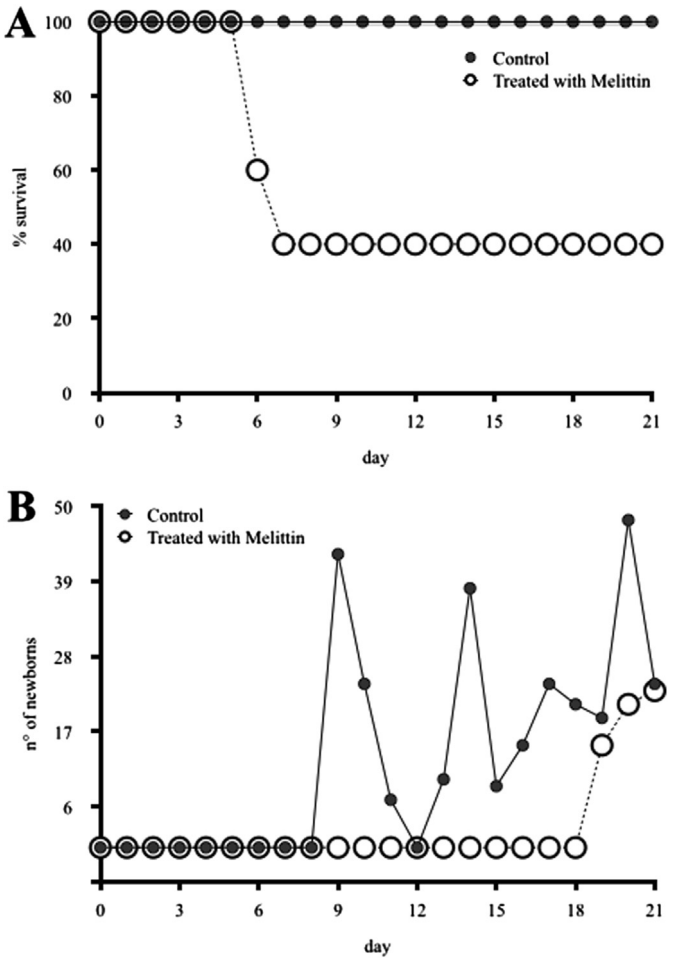

Fig. 2. Cumulative parent survival subjected to a chronic toxicity test for 21 days (A). Cumulative live offspring after 21 days of exposure to sublethal concentration of melittin (B).

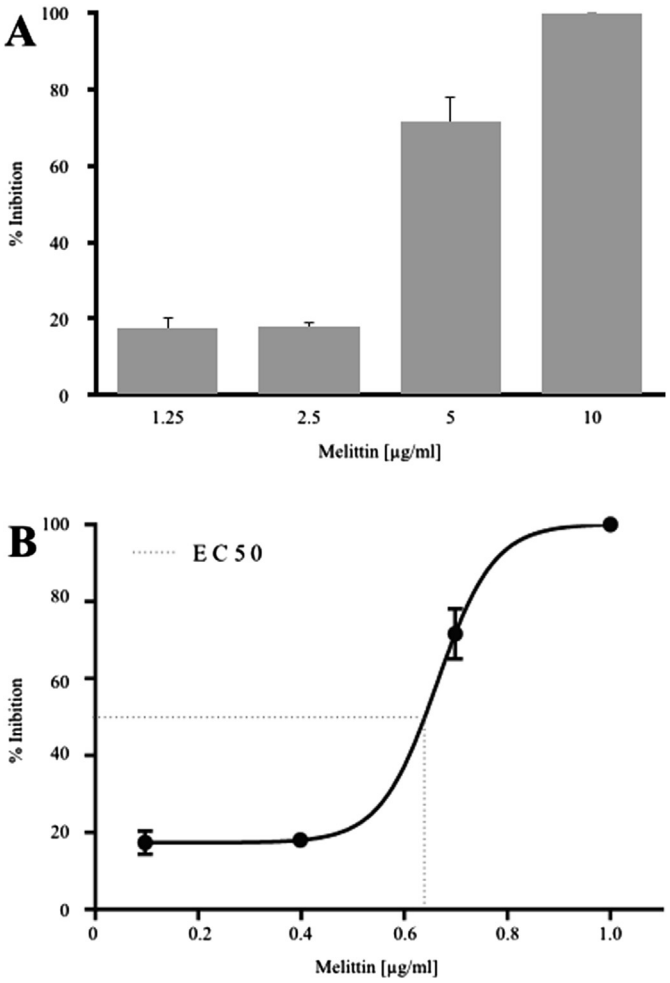

Fig. 3. Inhibition of growth of *P. subcapitata* submitted to several doses of melittin (A) and fitted curve by $\log_{10}$ from 72 h semi-chronic test (B) (IC_{50} 4.60 μg with IC 95% of 3.93–5.39; IC_{20} 3.8 μg with IC 95% of 2.3–6.29).

2.6.2. Comet assay to P. subcapitata

Protoplast isolation and purification were performed according to Gupta et al. (2011). For the comet assay, it was performed according to the method described by Kumar et al. (2011) with some modifications reported below. The protoplast solution (50 μL) was mixed with 50 μL of 1% low-melting temperature agarose dissolved in PBS. An 80 μL aliquot of the solution was layered onto a base slide, which was pre-coated with 1% agarose, and covered with a coverslip. The slides were submerged in alkali lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA) overnight at 4 °C. After, the glass slide was incubated in electrophoresis buffer in a horizontal electrophoresis tank for 30 min at room temperature for DNA unwinding (0.3 M NaOH and 1 mM EDTA, pH 13). Electrophoresis was performed for 10 min at 25 V and 300 mA in a chamber cooled in an ice bath. After electrophoresis, the glass slides were twice neutralized in 0.4 M Tris–HCl (pH 7,5) buffer and stained with 50 mL/mL DAPI (3 h).

2.7. Data analysis

The slides were examined on a fluorescence microscope (Leica DMLB microscope with digital camera Leica DFC340FX, Nussloch, Germany) and images were analyzed from each slide considering a minimum of 50 randomly selected nuclei. Comet images were captured from the center of the slide; overlapping figures were avoided. Quantitative assessment of DNA damage in selected nuclei was performed using Comet Score 1.5 Image Analysis (TriTek Corporation, Sumerduck, Virginia, USA) software, which computes the integrated intensity profile for each nucleus. The median of Tail Moment (TM) of 50 nuclei per slide was calculated for *D. magna* and the median of % of DNA in tail (%DNA) of 50 nuclei per slide was calculated for *P. subcapitata*. The software IBM SPSS Statistics® v.21 was used for statistical analysis. The distribution of TM and %DNA median values obtained from Comet Assay on untreated samples (controls) was analyzed by Shapiro–Wilk and Kolmogorov–Smirnov tests. To compare the results obtained from assays on samples subjected to different treatments, Levene's test was firstly applied to evaluate variance homogeneity. When homoscedasticity was verified, comparison was performed by ANOVA. In the case of non-homogeneous variances, Kruskal–Wallis non-parametric test was applied.

The statistical analysis applied to physiological responses and fitness parameters have been calculated with GraphPad Prism 6.

3. Results

3.1. Toxicity on D. magna

Melittin exhibited a considerably acute toxicity in *D. magna* since the first 24-h exposure at all the concentrations used in the

Table 1
DNA damage expressed as median, Standard Error (SE), and 75th percentile of Tail Moment in *D. magna* in cells exposed to different melittin concentrations.

		50 μg/mL	10 μg/mL	5 μg/mL	1 μg/mL	0.1 μg/mL	Control
Mobile	Median ± SE	12.10 ± 1.87	10.21 ± 1.44	9.14 ± 1.25	8.12 ± 1.31	5.91 ± 1.39	0.38 ± 0.30
	75th percentile	18.76	22.96	15.81	14.32	8.18	1.67
Immobile	Median ± SE	4.21 ± 1.04	4.74 ± 1.01	4.34 ± 3.50	8.18 ± 2.46		
	75th percentile	8.93	12.62		20.31		
Total	Median ± SE	4.99 ± 0.93	7.86 ± 1.00	9.02 ± 1.20	7.06 ± 0.79	5.91 ± 1.39	0.38 ± 0.30
	75th percentile	11.97	17.58	15.11	14.21	8.18	1.67

assay. From the analysis of regression curves of the acute toxicity data at 48 h, we obtained the EC_{50} of 38.49 μg/mL and EC_{20} of 5.535 μg/mL, which represent the lethal concentrations of melittin causing a *D. magna* mortality of 50% and 20% respectively (Fig. 1B).

The 21-day chronic test was performed on *D. magna* upon exposure to sub-lethal concentration of 1 μg/mL. The effect of exposition on survival and reproduction are shown in Fig. 2A. The survival rate and the reproduction times were evaluated. Our results showed that no mortality was observed for untreated daphnia until 21 days, while the survival of daphnids treated with 1 μg/mL of melittin was of 60% after 6–7 days and it decreased to 40% between 7 and 21 days although the contamination remained constant. This result suggests an adaptive response as reported for other toxicants (Kim et al., 2012).

Exposure to melittin also significantly impaired the reproductive output of *D. magna*. The treated group compared to the control group showed an inhibition of reproduction as well as a retardation of reproduction (Fig. 2B).

The assay also showed some long-term effects on reproduction; in fact, all treated daphnids have neonates after 21 days while control daphnids have neonates after 9 day and then every three days. A decrease on the mean number of neonates produced per female was observed at the end of the test. These results indicate that the reproductive parameters were very sensitive to treatments.

3.2. Toxicity on P. subcapitata

We observed a growth inhibition of the microalgae *P. subcapitata* after 72 h exposure to melittin with an IC_{50} at 4.6 μg/mL with an IC 95% of 3.2–4.2 and an IC_{20} of 3.8 μg/mL with an IC 95% of 2.3–6.2. Significant differences from the control growth rates were detected at the concentration of 10 μg/mL where the growth inhibition is almost 100% (Fig. 3A and B).

3.3. Genotoxicity

Genotoxicity is related to an increase of DNA damage as a consequence of the treatment with increasing concentrations of melittin.

To evaluate whether melittin exerted genotoxicity on *D. magna* and *P. subcapitata*, DNA damage and in particular DNA strand breaks were determined using a Comet assay. In fact, *in vitro* genotoxicity tests have gained increasing popularity as a tool supporting environmental risk assessment. The data demonstrated that treated samples were characterized by higher genotoxicity in comparison to untreated control samples. Tail moments increased for both bioindicators exposed to higher doses of melittin.

As shown in Table 1 and Fig. 4, Tail Moment increased significantly in cells isolated from *D. magna* that have been exposed to melittin at interval of concentration between 50 and 0.1 μg/mL, showing the occurrence of dose-dependent DNA damage. Median and 75th percentile were applied as suitable measures for highly skewed Tail Moment distributions. Median values for control samples were significantly lower than after treatment ($P < 0.001$ Kruskal–Wallis ANOVA rank model). We considered that analysis of median comet metrics from experiments at different exposure rate levels is certainly an efficient way to demonstrate a melittin genotoxic effect. Fig. 4 showed differences in baseline median TM values between the *D. magna* cells after treatment, which in most cases were confirmed through visual evaluation of boxplot graphs. Samples in 50 μg/mL, 10 μg/mL, 5 μg/mL, 1 μg/mL, 0.1 μg/mL produced significant amount of damage when compared with control. There was a clear dose-dependent response with increasing values of TM accompanying melittin concentrations. Nonparametric test was used to evaluate the difference in time-reliable cell responses for different melittin concentrations. We found a statistically significant difference between the TM of each population and the

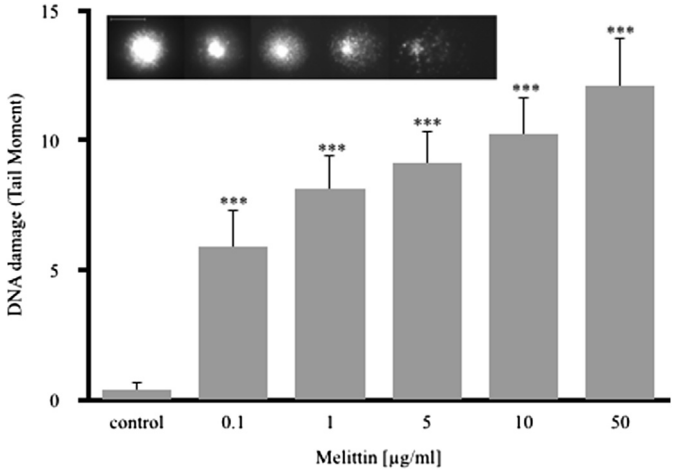

Fig. 4. DNA damage expressed as Tail Moment in *D. magna* in cells exposed to different melittin concentrations. (*** indicate $P < 0.001$).

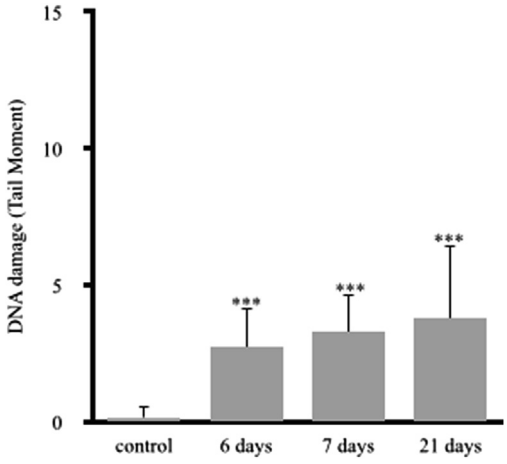

Fig. 5. DNA damage, expressed as Tail Moment, in *D. magna* in cells, exposed to 1 μg/mL of melittin concentration for 21 days. (*** indicate $P < 0.001$).

Table 2
DNA damage expressed as median, Standard Error (SE), and 75th percentile of Tail Moment in *D. magna* in cells, exposed to 1 μg/mL of melittin concentration for 21 days.

	1 μg/mL after 6 days	1 μg/mL after 7 days	1 μg/mL after 21 days	Control
Median ± SE	2.74 ± 1.39	3.30 ± 1.38	3.77 ± 2.66	0.16 ± 0.40
75th percentile	8.04	7.68	9.91	0.42

control ($p < 0.001$, Kruskal–Wallis test (Lovell and Omori, 2008), made one by one population versus control). In particular, a statistically significant increase of DNA damage was observed at a melittin dose of 50 μg/mL in both mobile and immobile daphnids; for the mobile daphnids there is a linear correlation with the dose while for the immobile ones the DNA damage is highly variable. A significant difference in DNA damage was detected at low concentrations of melittin in the acute toxicity test when we had an immobilization of daphnids of approximatively 20%.

The results obtained by exposing daphnids to a sublethal dose of melittin for 21 days are shown in Fig. 5 and Table 2. During a chronic experiment in which *D. magna* were exposed for 21 days to 1 μg/mL of melittin, statistically significant correlations were observed with an increasing DNA damage over time which were always higher compared to the control group (Kruskal–Wallis test, $P < 0.001$). High values of SE and 75th percentile for samples after 21 days indicate a high level of DNA damage.

The comet assay was performed also for *P. subcapitata*; the

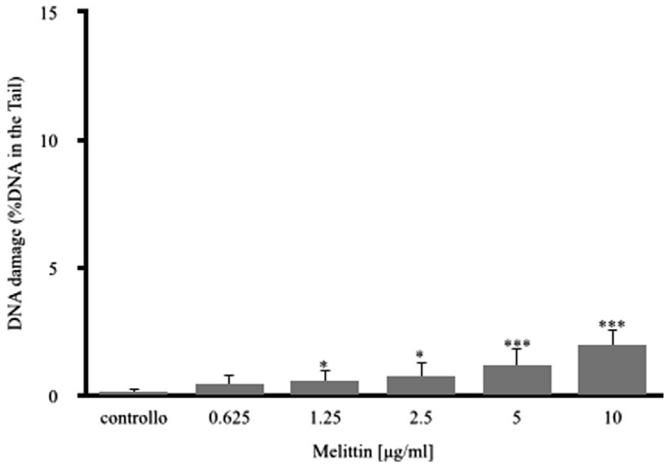

Fig. 6. DNA damage expressed as %DNA in the Tail in *P. subcapitata* in cells exposed to different melittin concentrations. (*** indicate $P < 0.001$; * indicate $P < 0.05$).

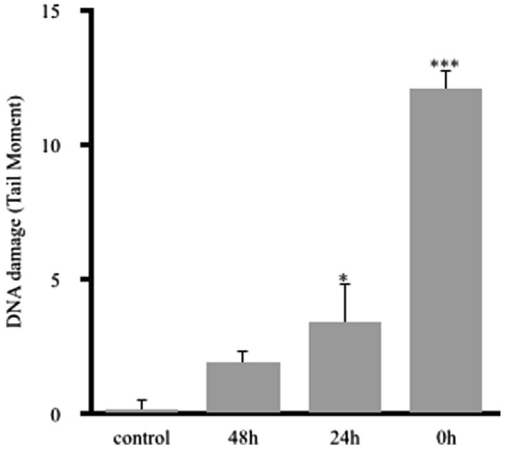

Fig. 7. DNA damage, expressed as Tail Moment, in *D. magna* in cells exposed for 48 h to 50 μg/mL melittin concentration, and the subsequently recovery after 24 h and 48 h from the treatment (*** indicate $P < 0.001$; * indicate $P < 0.05$).

Table 3
DNA damage expressed as median, Standard Error (SE), and 75th percentile of %DNA in the Tail in *P. subcapitata* cells exposed to different melittin concentrations.

		10 μg/mL	5 μg/mL	2.5 μg/mL	1.25 μg/mL	0.625 μg/mL	Control
Mobile	Median ± SE	2.16 ± 0.56	1.35 ± 0.67	0.95 ± 0.54	0.75 ± 0.43	0.65 ± 0.34	0.34 ± 0.15
	75th percentile	9.81	6.71	5.26	4.64	4.34	1.33

Table 4
Coefficient of correlation among observed parameters in *D. magna* and *P. subcapitata* exposed to melittin (Pearson correlation analysis indicates statistically significant differences between our samples and control; $p \leq 0.1$*, $p \leq 0.01$**, $p \leq 0.001$***, $p \leq 0.0001$****; r = pearson; R^2 = coefficient of determination).

	% Immobilization vs. DNA damage (crustacean)	% Inhibition vs. DNA damage (algae)	% Immobilization vs. % Inhibition	DNA damage (crustacean) vs. DNA damage (algae)
r (95% CI)	0.9388(0.3291 to 0.9961)	0.9642 (0.5486 to 0.9977)	0.9919 (0.8784 to 0.9995)	0.9216(0.2107 to 0.9949
R^2	0.8813	0.9297	0.9839	0.8494
p value	0.0090**	0.0040**	0.0004***	0.0130*

Table 5
DNA damage expressed as median, Standard Error (SE), and 75th percentile of Tail Moment in *D. magna* in cells exposed for 48 h to 50 μg/mL melittin concentration, and the subsequently recovery after 24 h and 48 h from the treatment.

	50 μg/mL	50 μg/mL 24 h after treatment	50 μg/mL 48 h after treatment	Control
Median ± SE	12.10 ± 1.87	3.41 ± 1.44	1.92 ± 0.41	0.15 ± 0.39
75th percentile	18.76	6.36	2.92	0.42

results clearly show that melittin induced DNA damage in a dose-dependent manner. The damage is low with only about 75% of DNA in tail (Fig. 6, Table 3) and a lower genotoxicity if compared to *D. magna*.

The correlation between geno- and ecotoxicity parameters was analized, using the Pearson test, in order to validate the ecotoxicological relevance of the response of DNA damage due to melittin exposure in *D. magna* and *P. subcapitata* (Table 4). Significant correlations were observed between DNA damage and mortality due to exposure to melittin.

3.4. Antigenotoxicity

We also monitored the DNA repair process in injured daphnids following high concentration of melittin (50 μg/mL); we observed that already after 24 h but even more after 48 h from removal of melittin, there was a significant reduction in genotoxic effects which returned to values close to control group (Table 5 and Fig. 7). Therefore, we can envision that the DNA damage is associated to the presence of the peptide. Statistical difference was observed when comparing TM values after treatment at 50 μg/mL to control samples. After 24h and 48h we observed lower values in TM related to treatment at 50 μg/mL, and differences with control samples are not statistically significant. Cells exposed to melittin showed lower DNA damage, slower repair, and higher residual unprepared damage after treatment. The decrease in DNA strand breaks in cells exposed to melittin can be visualized in Fig. 7 and Table 5 (*** indicate $P < 0.001$, *indicate $P < 0.05$). However TM even after 48 h repair time was not significantly higher (Kruskal–Wallis test, $P > 0.05$) than TM values for control group. This result may suggest that melittin caused an irreversible change at a molecular level, but DNA damage was reduced significantly and DNA damage repair mechanisms were accelerated.

4. Discussion and conclusion

Antibiotics effectively reduce the occurrence of infectious diseases but their widespread and often indiscriminate use has led to the rapid emergence of multi-drug resistant bacteria and a difficult control of these pathogens with the existing classes of antibiotics. Therefore, there is an urgent need to develop new classes of antimicrobial agents like antimicrobial peptides (AMPs), which showed to be suitable as an alternative class of potential therapeutics.

In this study, eco-toxicity and genotoxicity of melittin were investigated in the aquatic sentinel species *D. magna* and in the algae *P. subcapitata*. *D. magna* is one of the most popular herbivorous cladocerans considered to be a relevant test organism due to the feasibility of a short-term study for assessing ecotoxicity. The algae *P. subcapitata*, among the aquatic organisms, plays a key role due to its fundamental participation in energy conversion and ecosystem food web maintenance in the food chain of aquatic environments. These two organisms are particularly indicated for the monitoring of aquatic environments. Acute and chronic toxicity tests are rapid and sensitive tools for the risk assessment of novel potential therapeutic molecules. Moreover, genotoxicity and antigenotoxicity are also gaining increasing popularity as a tool supporting environmental risk assessment. Considering the potential of *D. magna* and *P. subcapitata* as bioindicators species, and the importance of genotoxic biomarkers in ecotoxicity monitoring, measurement of DNA damage in these species could provide useful information for freshwater monitoring and risk assessment.

We evaluated the ecotoxicological and genotoxic risks correlated to the antimicrobial peptide melittin, because recently research is strongly involved in the development of novel molecules that could substitute or adjuvate antibiotics in the fight against bacterial diseases. Although the use of AMPs is still in its infancy before developing them as novel drugs it is fundamental to assess their ecotoxicity.

Few data are already available about melittin ecotoxicology. Moreover, evaluation of genetic toxicity using comet assay has been performed on numerous wildlife organisms, but few study have been conducted on daphnids and algae.

The acute toxicity assays on *D. magna* allowed us to conclude that melittin caused immobilization of 55% of daphnids at a concentration of 50 μg/mL after 48 h, and we obtained an EC_{50} of 38.49 μg/mL with IC of 22.29–66.45.

In the semi-chronic test of algae inhibition at lower concentration of melittin, we found an inhibition of growth of 80% at a concentration of 20 μg/mL with an IC_{50} of 3.7 with IC 3.2–4.2. Statistical analysis of comet assay showed that there was a significant difference between cells exposed and control, which indicates that melittin caused ecotoxic and genotoxic effect at lower doses.

It is remarkable that when we exposed *D. magna* to a sub-lethal concentration of melittin for 21 days in the chronic toxicity test, survival and reproduction decreased; while the DNA-damage with IC of 2.74–3.77 is lower than that observed in the acute test at the same concentration. This was in agreement with several other studies using various toxicants.

Our main finding is that melittin is substantially detrimental to *D. magna* reproduction and its sub-lethal concentrations create an accumulation dependent on exposition times and a negative effect on DNA. We also observed that melittin significantly delayed time to first eggs, which appears in brood chamber (pouch) at day 10. The response of *D. magna* and *P. subcapitata* to melittin in terms of mortality, surviving, and reproduction may well explain the consequences on the DNA. Sensitive detection of DNA damage in wildlife species is fundamental; in fact, pollutant-induced DNA damage might influence the genetic constitution of populations. The experiments with melittin-exposed *D. magna* show that there is a dose–response toxicity with about 60% of immobilization and long-term effects on reproduction and survival. The evidence that DNA damage occurred concomitantly with the decrease in these parameters suggest that DNA alteration might provoke quite high level consequences.

In conclusion, our results showed that melittin exerted its toxic and genotoxic effects in both species, being a bit more aggressive towards *P. subcapitata*. The dose usually used in *in vivo* experiments is of 20 μg/mL, and thus the dose which can get into the environment is much lower. In this study, we have demonstrated that this concentration does not induce toxicity to exposed cells.

Conflicts of interest

The authors declare that they have no competing interests.

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