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METHOD FOR COLLECTING COELOMOCYTES FROM *EURYTHOE COMPLANATA* (ANNELIDA: AMPHINOIDAE) WITH POTENTIAL APPLICATION IN TOXICOLOGY

METODO PARA COLECTAR CELOMOCITOS EN *EURYTHOE COMPLANATA* (ANNELIDA: AMPHINOIDAE) CON POTENCIAL APLICACIÓN EN TOXICOLOGÍA

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ABSTRACT

Benthic organisms have been proposed as good indicators of disturbance in marine environments, particularly because most toxins fall to the bottom of the sea affecting benthic communities. A non-invasive method for collecting coelomocytes was developed using the marine polychaete *Eurythoe complanata* with potential applications in xenobiotic toxicology. The extrusion method used produced the expulsion of 95% of the coelomic fluid, showing a greater efficiency than the puncture method in *Lumbricus terrestris* with regard to the total number of coelomocytes and cellular viability. The total coelomocyte count by this method was 2.6 x 10^6 cells·mL^{-1} *vs* 1.4 x 10^6 cells·mL^{-1} for the puncture method. The results indicate that coelomocyte numbers completely recover at 20 days after extrusion, using the total cell count, cell viability and differential count. This method was developed to study immunotoxicity of xenobiotics at the sediment-water interface of marine environments.

Keywords: Coelomocytes, *Eurythoe complanata*, Immunocompetition, Polychaete.

RESUMEN

Los organismos bentónicos han sido señalados como buenos indicadores de alteraciones en el medio marino, particularmente porque la mayoría de los contaminantes se van al fondo marino, afectando a las comunidades que allí habitan. En el presente trabajo se desarrolló un método no invasivo para colectar los celomocitos usando el poliqueto marino *Eurythoe complanata* con potencial aplicación en toxicología. El método de extrusión utilizado provocó la expulsión del 95% del fluido celómico, mostrando una mayor eficiencia que el método de puntura en *Lombricus terrestre* en relación al número de celomocitos y la viabilidad celular. El conteo total de celomocitos por este método fue 2.6 x 10^6cells·mL^{-1} *vs* 1.4 x 10^6cells·mL^{-1}para el método de puntura. Los resultados indican una completa recuperación en el número de celomocitos a los 20 días posteriores a la extrusión; utilizando el conteo celular total, viabilidad y conteo diferencial celular. Este método fue desarrollado para estudios de inmunotoxicidad de xenobioticos en la interfase sedimento-agua de ambientes marinos.

Palabras clave: Celomocitos, *Eurythoe complanata*, Immunocompetencia, Poliquetos.

INTRODUCTION

Organisms have developed mechanisms that allow them to adapt to the different habitats in which they live. One of the most important of these is their immune system which has been essential for the survival of many species. In invertebrates, the plasticity and effectiveness of their immune system as a vital mechanism of protection has allowed them to defend themselves from xenobiotic attacks, so that they have been able to survive and colonize most kinds of habitat (Galloway & Dpledge 2001, Antón & Salazar 2009). Aside from being one of the main defence mechanisms of the organism the immune system is also the first to respond to the presence of strange agents, including toxins and chemicals. Methods using the immune response have thus been developed for screening chemicals and assessing their mode of action on terrestrial and aquatic organisms. The use of immune systems has been demonstrated for assessing toxicity under both acute and chronic conditions of exposure (Anderson & Chain 1982). Since certain aspects of the functions of the immune system are phylogenetically conserved, it is possible to use the immune responses of several invertebrate taxa in the development of immunological biomarkers, to assess the toxic effects of environmental contamination (Anderson & Chain 1982, Anderson *et al.* 1992, Zapata-Vivenes *et al.* 2005).

Currently, immune responses are being developed to evaluate the toxicity of nanomaterials (Borm *et al*. 2006, Zolnik *et al*. 2010). Several standardized immunoassays have been developed using earthworms for measuring the immunotoxic potential of xenobiotics in mammals. These are based on cell-mediated, humoral and nonspecific responses, namely wound healing, secretory erythrocyte formation and phagocytosis by earthworm leucocytes (coelomocytes). In addition, immunocompetence has commonly been assessed by the collection of coelomocytes by puncture of the coelomic cavity (Eyambe *et al.* 1991, Goven *et al*. 1993).

Like earthworms, polychaete worms may well be model organisms for revealing immunological threats posed to marine fauna from subtle chemical contamination in the benthos. These organisms are useful sentinels for benthic pollution (Casado- Martínez *et al.* 2012, Lewis & Walson 2012) and possess immunological elements similar to those of earthworms suggesting their potential for the development of adequate and sensitive marine immunotoxic screening protocols. Here, we describe a non-invasive extrusion method for harvesting coelomocytes from the polychaete *Eurythoe complanata* (Pallas 1766) (Annelida: Amphinoidae) *with* minimal trauma to the worms, which could be potentially useful for immuno-toxic studies. We compared the extrusion and puncture methods by measuring cell yield and viability.

The frequency at which coelomocytes could be sequentially sampled without affecting either their total and differential counts, or their viability, was also ascertained.

MATERIAL AND METHODS

Specimens of *E. complanata* (worms) were collected from the Gulf of Cariaco, in northeastern Venezuela. The worms were maintained for two weeks prior to experimentation in aerated aquariums containing seawater (36%, 25± 1°C and pH 7.8) and sand/gravel from the collection site in order to provide food and shelter.

A non-invasive extrusion method for the obtention of coelomic fluid

Coelomocytes were obtained from coelomic fluid harvested by a non-invasive extrusion procedure based on a previously developed method for earthworms used in

immunotoxicity studies. Worms weighing 1.0 ± 0.2 g were placed in a 10 cm diameter Petri dish containing 38-40 mL of one of several extrusion mediums, which were then tested for their efficacy in promoting the expulsion of coelomic fluid, whilst maintaining cell yield and viability. The extrusion mediums tested were prepared by mixing one of three concentrations of guaiacol glyceryl ether (Sigma Chemical Co. USA) (1, 5, 10mg/ mL) with varying concentrations of chloral hydrate (Merck) (50, 5, 2.5, 0.5, 0.05 mg/mL) in millipore-filtered (0.42 µm) seawater (pH 7.8) containing 2.0 mmol Na_2EDTA.

After 10 min. of exposure to the extrusion solution, the irritant chloral hydrate induced a contraction of the body length to less than 60% of its initial size resulting in the extrusion of coelomic fluid. At this point, the worms were taken out of the Petri dishes and placed over paper towels wetted with seawater to collect the fluid directly from the pygidial pore by suction with a Pasteur pipette. Worms were harvested on day 0 (initial extrusion) and subsequently on days 3, 7, 15 and 20 of the experimental period.

Puncture method for coelomocyte collection
Coelomic fluid was removed from the worms by inserting a sharpened Pasteur pipette into the coelom and allowing the pipette to fill by intracoelomic pressure.

Collection of coelomocytes
Aliquots of concentrated fluid (0.1-0.25 mL) taken from each worm were transferred to small plastic test tubes (ependorf) and subjected to centrifugation at 200 x g for 10 min. The resulting pellets were washed twice and then re-suspended in 1 mL of seawater (pH 7.8 at 4ºC) containing 0.01 mmol EDTA (Ethylendiamine-tetraacetic acid disodium salt, Sigma Chemical, Co., St Louis Mo., U.S.A.). The cell suspension was then adjusted to $1x10^6$ cells·mL^{-1}.

Coelomocyte counts and viability
Total coelomocyte counts from each worm were made using a Neubauer haemocytometer and expressed as the mean ± standard deviation per mL. Viability was examined by staining 0.4 % Trypan blue (Sigma Chemical Co) in millipore filtered (0.22 µm) seawater and reported as the percentage of live cells at the time of counting. Differential cell counts were recorded after smearing a drop of coelomocyte suspension on a glass slide and staining with Wright-Giemsa (Sigma Chemical. Co). One hundred coelomocytes were identified as basophils, acidophils or transitionals under oil immersion microscopy at 100X, according to the method described by Hostetter & Cooper (1974).

Data analysis
The bioassay results were analyzed statistically using ANOVA, LSD test as posteriore and contingency tables (Sokal & Rohlf, 2012).

RESULTS AND DISCUSSION

Exposure of the polychaetes to 2.5 mg/mL of chloral hydrate combined with 1 mg/mL guaiacol glyceryl ether provoked a rapid irritation response associated with a powerful contraction of the body resulting in the expulsion of coelomic fluid through the pygidial pore (Fig. 1). At lower chloral hydrate concentrations (0.05 and 0.5 mg/ mL) only an anaesthetic effect was observed, while values higher than (5 and 50 mg/mL) were lethal. Guaiacol glyceryl ether exerted an effective antimucolytic action on the coelomic fluid at every tested concentration (1, 5 and 10 mg /mL). Based on these observations, we chose the extrusion medium of 2.5 mg/mL chloral hydrate and 1.0 mg/mL guaiacol glyceryl ether in millipore-filtered seawater (pH 7.5-7.8) supplemented with 1 mmol EDTA to minimize calcium induced cell aggregation in the

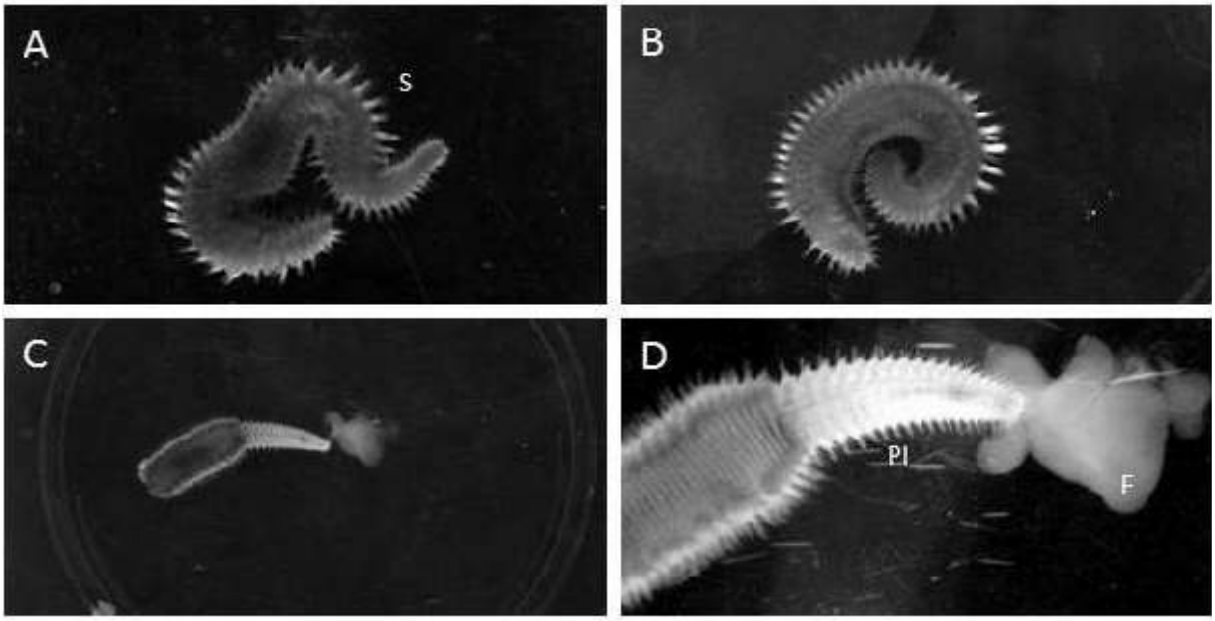

Figure 1. Sequence of extrusion process of coelomic fluid from *E. complanata*.
A: Initial stage extrusion.S: Extrusion medium
B: Sinusoidales movements.**C:** Body length reduction.
D: Coelomic fluid expulsion.
PI: Pigidie pore.
F: Coelomic fluid.

collected coelomic fluid, for the remainder of the study. The total counts of extruded coelomocytes were significantly higher than those sampled by the puncture method (ts= 6.10; P< 0.01). The differential distribution of coelomocytes (X^2= 0.019; P> 0.05) and the differential cellular count (DCC: basophils 40%, acidophils 51% and transitionals 7%) were essentially similar for both collecting procedures, this last analysed using a contingency table (X^2: 0.019, P< 0.0995, gl: 2). Basophils and acidophils were nearly equally represented in the cell population, whereas transitional or immature coelomocytes were less abundant.

The total counts, viability and coelomocyte profiles for polychaetes subjected to repeated extrusion of the coelemic fluid indicate that approximately 3 weeks are required for coelomocyte numbers to completely recover after extrusion. Total counts varied significantly among samples taken over a 20 d period (ANOVA: F= 8.66, P< 0.01; Fig. 2). An LSD test indicated, however, that initial and final counts did not significantly vary. Coelomocyte viability significantly changed between repeated extrusions, being at minimal levels during the first week after the initial extrusion, but thereafter quickly recovering normal numbers (ANOVA: F=70.87; P<0.01; Fig. 2).

The proportions of the components of the coelomic cell population varied significantly over the course of the 20 d. extrusion assay (ANOVA: basophil F= 9.92; P< 0:01; acidophil F=4.72; P<=.0.01; transitional F= 50.15; P= 0.01; Fig. 2). However, no significant differences were found between mean values at the beginning (collection day 3) and at the end (collection day 20) of the experimental period, for basophils and acidophils.

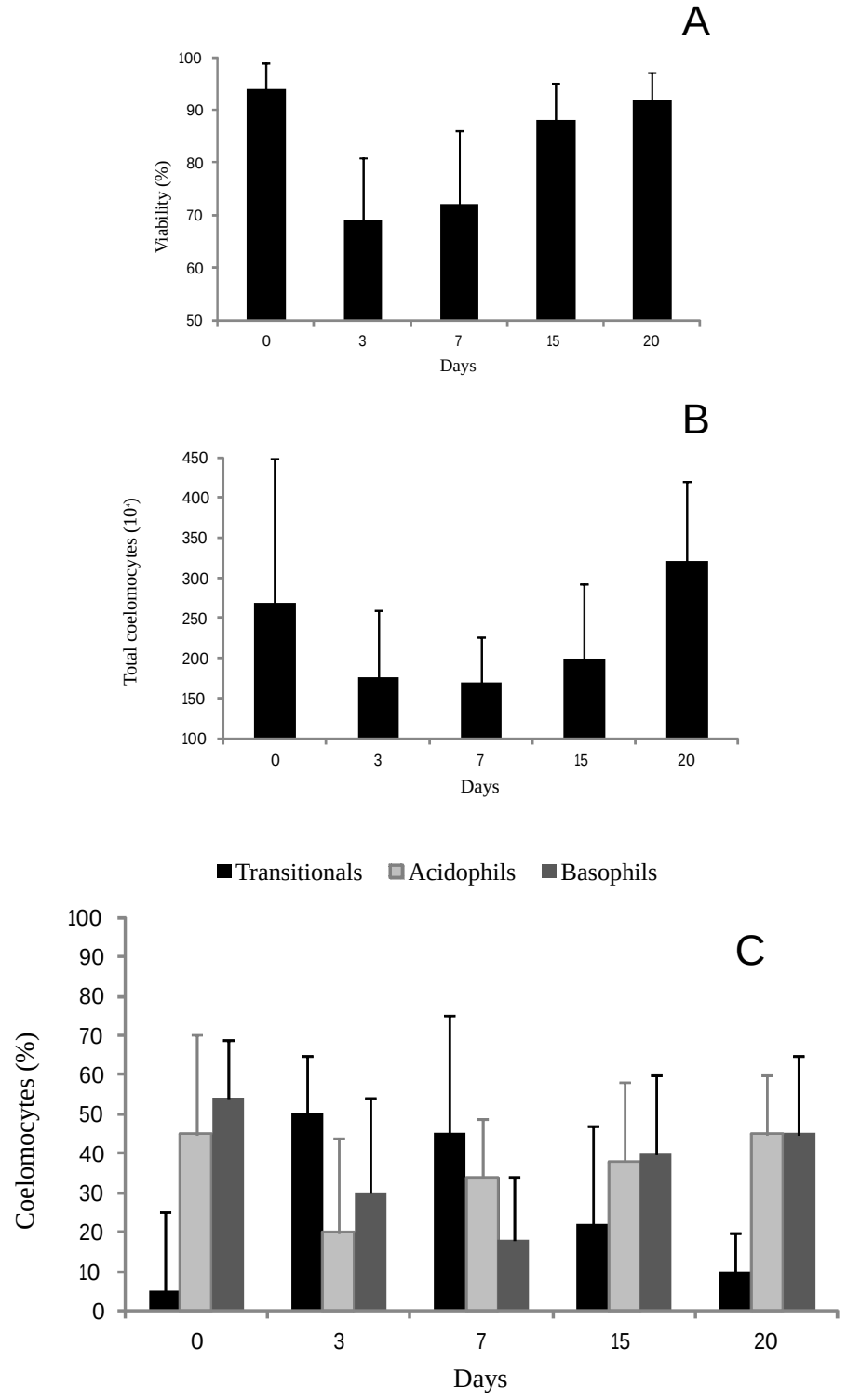

Figure 2. **A.** Cell viability in relation to days of recovery from *E. complanata*. **B.** Total number of coelomocytes in relation to days of recovery from *E. complanata* **C.** Comparison of differential cell, classified: (basophils, acidophils and transitionals) in relation to days of recovery from E. *complanata*

We have developed a method for the extrusion of coelomic cells. This method succeeded in 95% of cases, in contrast with the method described by Peter in which a success rate of only 60-80% was obtained (Goven *et al.* 1993). This difference in efficiency could be due to differences in the chemical composition of the extrusion medium used Extrusion methods are superior to puncture methods, because they enable the collection of a higher number of cells (2.6×10^6 cells·mL^{-1}vs. 1.2×10^6 cells·mL^{-1}).

The extrusion method we used improves upon the one described by Eyambe *et al.* (1991) by inducing the release of the fluid from a single pore. Possibly, owing to differences in the species, the extrusion period of our method (6-14 min.) was substantially higher than that of Goven *et al.* (1983).

Regarding cellular viability and differentiation, the two methods (puncture and extrusion) function equally well. The results indicate that 94% of cells are viable, a similar value to that reported for annelids (Frolich *et al.* 1982, Jacobs *et al.* 1983, Goven *et al.* 1983). The decrease observed in cellular viability between three and seven days may be attributed to the presence of immature cells and possibly very fragile coelomocytes. Cellular viability recovered, however, between 15 and 20 days (Fig. 2).

The counts of basophils obtained in the first extrusion were similar to these reported by Toupin & Lamoureux (1976). Basophilic cells, have been identified as the most immunoactive coelomocytes (Liebman, 1942; Hostetter & Cooper 1974; Stein *et al.* 1977; Cooper & Stein, 1981). The proportion of basophilic and acidophilic cells followed a similar time-course, which was inversely correlated with the numbers of the transitional cell population. In annelids transitional cells are immature cells (Liebman 1942, Hostetter & Cooper 1974), precursors to the basophilic and acidophilic cells. These cellular groups are thus related such that the increase or decrease in one group affects the proportions of the others.

The method to obtain coelomocytes we outlined here, shows a number of experimental advantages that could facilitate the development of protocols for assessing xenobiotic immunotoxicity (Zapata-Vivenes *et al.* 2005). *E. complanata* is an important species in the benthos and is broadly considered as a potential sentinel for detecting sediment pollution. This paper establishes a simple method for the collection of coelomocytes from *E. complanata* that gives satisfactory results for use in immunological studies while minimizing the negative effects of puncture. This method gives cells that are free of tissue fragments and allows recovery of the organisms at 20 days post extrusion.

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