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Immunodetection of *Mytilus galloprovincialis* larvae using monoclonal antibodies to monitor larval abundance on the Galician coast: Optimization of the method and comparison with identification by morphological traits

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ABSTRACT

The method currently used for accurate identification of mussel larvae is based on the study of morphological traits under an optical microscope, which is a tedious and time-consuming procedure. It also requires considerable taxonomic experience, because of the similarities in the larvae of different bivalves present in the plankton. The introduction of specific monoclonal antibodies (mAbs) directed against mussel larvae, such as M22.8 and M36.5 mAbs developed by our group, may allow an easier and more specific identification. Handling conditions and sample preservation were optimized for using these antibodies in the monitoring of mussel larvae in the Galician rías. Bivalve larvae can be isolated very efficiently from plankton samples by centrifugation in sugar solution. Samples can be maintained at 4 °C on the boat and during transport to the laboratory, and then preserved for longer periods at −80 °C or in liquid nitrogen until staining. In an attempt to minimize the time required for immunodetection, different incubation periods were tested, which showed that only 5 min of incubation with the primary monoclonal antibody and 60 min with the secondary antibodies are sufficient to stain over 98% of the larvae. Here, we show that the use of mAbs allows a rapid and specific recognition of mussel larvae, with clear advantages over the traditional method, particularly for large-scale field studies.

1. Introduction

Spain is the third largest producer of mussels in the world (FAO, 2006), while Galicia (NW of Spain) is the most important European region for their culture (Labarta and Pérez Corbacho, 2004). Mussel culture in the Galician rías is based on the on-growing of young individuals (seed) attached to ropes hanging from rafts located inside the estuaries (Pérez Camacho et al., 1991). The seed is obtained either directly from the natural populations of the intertidal zone, or from the plankton using spat-collecting ropes hung from commercial rafts. The latter method has greater advantages than the former. Firstly, mussels obtained in this way are larger at harvest time (Fuentes et al., 1998). Secondly, there is a reduction of the dangerous, laborious and socially conflictive removal of mussel seed from intertidal areas (Fuentes and Molares, 1994). Thirdly, interference with the extraction of other intertidal resources, such as the highly valued barnacle *Pollicipes cornucopia* (Molares and Fuentes, 1995), is avoided. To advise mussel farmers about the better areas of the rías and moments in the season to hang the spat-collecting ropes, the Galician Department of Fisheries and Maritime Affairs has established routine monitoring of the spatial and temporal distribution of the abundance of mussel larvae within the Galician rías. This monitoring involves weekly sampling of the water column at thirteen fixed stations in the rías, and requires a rapid and precise identification method to differentiate mussel larvae from the other bivalve larvae present in the samples. The current method for the identification of bivalve larvae is based on the microscopic observation of the morphological characters of the shell of each individual (reviewed in Lutz, 1985), which is a tedious and time-consuming process. To design a faster and more rigorous identification method, two monoclonal antibodies (mAbs) M22.8 and M36.5 directed against mussel larvae were generated in our laboratory (Abalde et al., 2003). We have shown that these two monoclonal antibodies stain all larval stages and permit the specific identification of mussel larvae in plankton samples by immunofluorescence assays (Lorenzo-Abalde et al., 2005). Therefore, the incorporation of these two specific antibodies into larval monitoring would permit not only a faster and more precise identification of the mussel larvae, but would also allow an increase in the number of sampling stations to be analysed each week.

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However, before this can proceed, some of the steps in sample management of the current monitoring procedure should be modified to make them compatible with the use of these new immunological tools. Firstly, the current method of sample preservation, using 4% buffered formalin–seawater solution, must be changed, as formalin can cause loss of antigenicity, due to the induction of chemical changes in the antigen epitopes (Leong and Gilham, 1989; Shi et al., 2007), or antigenic masking (Brandtzaeg and Rognum, 1984). Therefore, we have tested and compared different sample preservation procedures for plankton samples, in an attempt to find a suitable and compatible method with the immunofluorescence assay. Secondly, samples taken in the water column of nutrient-rich ecosystems, such as the Galician rías, contain a high variety of planktonic species of different shapes, sizes and pigment composition. The presence of these species in the sample could disturb the detection, and affect the counting of mussel larvae in the immunofluorescence assays, either due to the autofluorescence produced by the pigments of some species, or simply due to physical interposition. Hence, our purpose was to test an easy-to-use and efficient method for the separation of bivalve larvae from other planktonic species present in the samples. Thirdly, time is a critical aspect of larval monitoring, as it requires the processing of a large number of samples in just two or three days. The most time-consuming steps of the immunofluorescence assay are the incubations of the bivalve larvae with the primary and secondary antibodies. To reduce these steps to a minimum, a series of incubation times was tested with both antibodies. Once all steps of sample management were adapted, several plankton samples taken from the Arousa ría (Galicia, Spain) were processed using the two methods: the modified one based on immunodetection, and the current one based on identification by morphological traits. Finally, the number of mussel larvae detected by the two methods was compared using a statistical test.

2. Materials and methods

2.1. Preservation of samples

Five different sample preservation methods were compared: ethanol 70%, glutaraldehyde 2.5%, 4% buffered formalin–seawater solution, refrigeration in seawater at 4 °C and freezing to −80 °C. For the comparison, five samples were used, each of approximately 2000 D-shaped mussel larvae taken from a common larval monoculture batch. Monoculture of D-shaped mussel larvae was carried out following the method previously described in Abalde et al. (2003). Three samples were introduced into bottles containing 20 mL of the respective chemical preservative (ethanol, glutaraldehyde or formalin) and one into a bottle maintained at 4 °C containing 20 mL of 0.20 µm filtered seawater. The fifth sample was transferred into a cryotube with 1 mL of filtered seawater and 10% dimethyl sulphoxide (DMSO) (Panreac, Barcelona, Spain) as cryoprotector, and then frozen to −80 °C. After 24 h larvae were rinsed three times in distilled water to remove the fixative. The frozen larvae were previously thawed in a 37 °C bath, and a replicate of larvae maintained at 4 °C was left in distilled water for 45 min, before the immunofluorescence assay. To avoid possible unspecific binding due to the fixative method, larvae were preincubated with phosphate buffered saline (PBS: 0.15 M ClNa, 2.7 mM ClK, 1 mM Na_2HPO_4, 1.8 mM KH_2PO_4), gelatine 3% (Panreac) for 30 min. Three subsamples were taken from each preservation method and, subsequently, subjected to indirect immunofluorescence assay. For each fixative method, samples were incubated with 600 µL of hybridoma supernatants containing M22.8 or M36.5 mAbs, or with PBS as negative control. After 120 min of incubation, larvae were washed three times with PBS-1% bovine serum albumin (BSA) (Amresco Inc., Ohio, USA) and incubated with fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulins (Caltag, Invitrogen Corporation, Carlsbad, California), diluted 1/800 in 600 µL PBS-BSA 1% for 120 min and, subsequently, washed three times with PBS-BSA 1%. Finally, the percentage of stained larvae for each sub-sample was determined using an epifluorescence inverted microscope (Olympus IX-50). The experiment was repeated three times.

2.2. Separation of bivalve larvae

Several plankton samples were collected at different points of the Arousa ría (NW Spain) by double vertical tows from 10 m deep to the surface, using a bongo net with a 40 µm mesh. Once on board, samples were kept cold in a camping refrigerator until arrival at the laboratory, where each sample was filtered using a 40 µm pore side length mesh, and resuspended in 20 mL of sterile seawater. The bivalve larvae were then separated from the rest of the planktonic organisms of the sample by centrifugation in a sugar gradient following, with minor modifications, the protocol used by Paugam et al. (2003) for the larvae of the scallop *Pecten maximus*. Therefore, a syrup was prepared with commercial sugar with a density close to 1.2 g/cm^3, as previously described by Tremblay et al. (1987) for bivalve separation, rather than one of 1.30 g/cm^3 that is specific for scallop. Each plankton sample was gently poured, drop by drop, into a 50 mL Falcon tube (Beckton Dickinson, USA) containing 25 mL of the sugar syrup and then centrifuged (PK12R, ALC, International SRL, Milan, Italy) for 2 min at 300 ×*g*. Following centrifugation, three phases and a pellet were observed in all tubes. Each phase and the

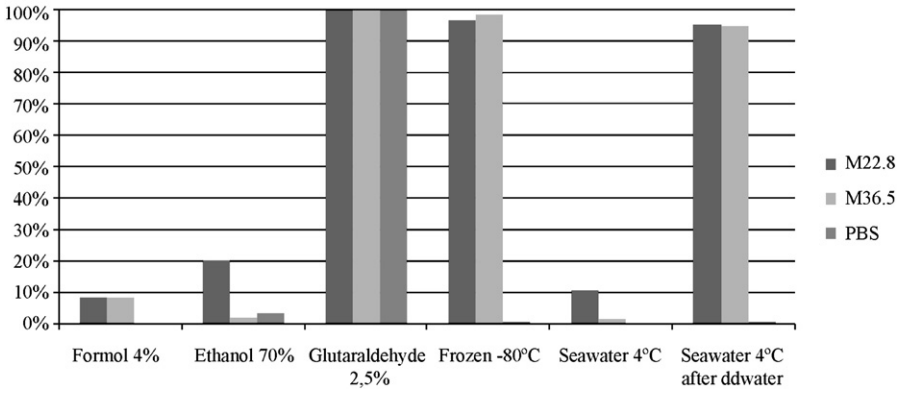

Fig. 1. Percentage of immunostained *Mytilus galloprovincialis* larvae (maintained or fixed using different methods). The M22.8 and M36.5 mAbs were used as primary antibodies, followed by FITC-conjugated secondary antibodies. Negative control omitting the primary antibody (PBS) was also included.