

# The micronucleus test: temporal pattern of base-line frequency in *Mytilus galloprovincialis*

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**ABSTRACT:** The base-line frequency of micronucleated cells was measured in gill tissue of *Mytilus galloprovincialis* Lmk. from 2 Mediterranean areas (northern Tyrrhenian and northern Adriatic Seas), and turned out to be a function of water temperature and age of the animals.

## INTRODUCTION

Micronuclei are secondary nuclei, formed during telophase, considerably smaller than the principal nucleus. They originate from chromatin lagged in anaphase as a consequence of chromosome breakage or malfunction of the spindle apparatus. In the first case they contain an acentric chromosome fragment, in the second an entire chromosome. Whichever the case, an increase in the frequency of micronucleated cells present in a tissue is an index of chromosome damage due to some genotoxic agent (Heddle et al. 1983, Landolt & Kokan 1983, Al-Sabti 1986a, b).

In a previous study, we showed that the micronucleus test on the gill tissue of *Mytilus galloprovincialis* Lmk. could be used to monitor environmental genotoxicity (Brunetti et al. 1988). This possibility was inferred from the fact that micronucleus frequencies induced by a clastogenic agent (mitomycin C) persist for a very long time after the end of treatment (Majone et al. 1987). We later also showed that an aneuploidizing substance (colchicine) has the same effect (Majone et al. 1990), confirming the validity of the test in environmental studies.

In the above-quoted examples of application of the test (Brunetti et al. 1988), samples collected at the same time from a specific basin or marine area were compared and we concluded that the station in which specimens revealed higher micronucleus frequencies must be considered as more polluted. However, a problem arises when it is wished to compare samples collected at different times and from different areas because the influence of season and age on the sensitivity of specimens cannot be excluded.

In this article we analyze this possibility by long-term sampling of controlled mussel populations living in 2 different sites in the Mediterranean.

## MATERIALS AND METHODS

**Sampling stations and specimens.** Two Mediterranean sites were chosen: the Portovenere Channel (off La Spezia, Italy) in the northern Tyrrhenian Sea, and the waters in front of the Castle of Duino, Italy (near Trieste) in the northern Adriatic. The pattern of water temperatures in the 2 sampling areas was similar, with values above 20 °C (up to 25 °C) from June to September and below 15 °C from October to April with winter minima of about 8 and 12 °C respectively. Both sites are recognized as suitable for aquaculture and are periodically monitored by Italian health authorities. Thus we considered these areas to represent unpolluted coastal waters. At both stations (depth 10 to 12 m) 1 yr old mussels were collected from suspended cultures and placed in 2 plastic nets fixed at depths of 1.5 and 4.5 m respectively. Fifteen mussels per net were sampled every 2 mo. Lengths of the major and minor axes of the shell and sex of individuals were recorded.

**Techniques.** The gills were removed and enzymatically digested in a solution of dispase-HBSS (0.60 to 1 U ml<sup>-1</sup>) for about 20 min at 37 °C, as suggested by Migliore et al. (1989). The resulting cell suspensions were fixed in methanol:acetic acid (3:1) and centrifuged at 1000 rpm for about 5 min. The pellets were resuspended in a small amount of fixative and a few drops were spread on slides, air-dried and stained with 5 %

Giemsa (Majone et al. 1988). A total of 2000 cells were scored for each mussel and micronucleus frequencies detected were compared statistically by means of the non-parametric Kruskal-Wallis and Wilcoxon tests (Sokal & Rohlf 1981).

## RESULTS AND DISCUSSION

### Growth

No difference was found between the sizes of the mussels (expressed as major and minor axis length of shell) collected at the 2 different depths. Table 1 reports mean sizes calculated on the pooled sub-samples. They did not show growth as a function of time. This may be due to the fact that samplings started when the mussels were about 1 yr old, while this species grows particularly fast in its first year of life. Consequently, the very slow growth which probably occurs after the first year of life is covered by sample variance. Lastly, no correlation could be found between the size and sex of individuals.

### Sex ratio

In *Mytilus* spp. sex may be determined only by observing the gonad. However, at the end of the reproductive season, the gonad regresses, making sex identification impossible. The time pattern of the frequencies of males, females and individuals with regressed gonads is shown in Fig. 1A, B. These data are in agreement with our knowledge of the reproductive biology of the species. In winter energy is transferred from stocked glycogen to the ripening gonads (Bressan & Marin 1985, Da Ros et al. 1985) and spawning takes place in spring. In summer a sexual rest follows,

although a fraction of the population may also be ripe. Our data indicate that ovaries regress before testes, and ripen later. Moreover, the data of Fig. 1B, which refer to mussels sampled for 2 yr, show that the summer rest in sexual activity is strongly enhanced in 2 yr old mussels as compared with that of 1 yr olds. This might indicate an effect of age on the summer regression of gonads. In winter, when all individuals are ripe, the sex ratio is 1:1.

### Micronucleus frequency

At both stations no statistically significant differences were found between frequencies detected in mussels collected at 1.5 and 4.5 m depth (data not shown). Thus, the 2 subsamples were pooled; the time pattern of the new mean frequencies is shown in Fig. 1A, B.

Comparing micronucleus frequencies detected during 1988 at the 2 stations, we note the same general trend and no significant differences in values. A highly significant increase in micronucleus frequencies was found in summer 1988. This may be a consequence of the thermal stratification which segregates pollutants in more superficial water layers, or a direct effect of temperature on the mitotic rate of mussels during their greatest period of growth. In the latter case the number of newly produced gill cells rises and, as a consequence, that of micronucleated cells too. It must be assumed that the mortality rate of micronucleated cells is approximately equal to that of normal cells. Although little is known about this, some recent data on Chinese hamster ovary (CHO) cells treated *in vitro* seem to support this hypothesis (F. Majone, S. Tonetto, C. Soligo & M. Panozzo unpubl.).

Data from the northern Tyrrhenian sea, where samplings continued in 1989, showed a decrease in micronucleus frequencies as a function of time, and

Table 1. *Mytilus galloprovincialis*. Mean length of major and minor axes of mussel shells (n = 30). Standard deviation in parentheses

Date	Tyrrhenian Sea		Adriatic Sea	
	Major axis (mm)	Minor axis (mm)	Major axis (mm)	Minor axis (mm)
3 May 1988	–	–	54.1 (6.1)	28.3 (2.2)
7 Jun 1988	65.6 (4.2)	36.5 (3.3)	–	–
4 Jul 1988	–	–	56.1 (5.9)	30.3 (2.7)
5 Aug 1988	65.1 (3.5)	35.9 (2.5)	–	–
9 Sep 1988	–	–	56.4 (4.7)	29.8 (2.2)
11 Oct 1988	66.9 (5.7)	36.8 (3.1)	–	–
15 Nov 1988	nd	nd	55.1 (4.2)	28.0 (2.6)
26 Nov 1988	65.8 –5.3)	40.9 (4.7)	nd	nd
1 Feb 1989	nd	nd	55.4 (4.1)	29.6 (2.4)
7 Mar 1989	64.9 (6.4)	34.6 (2.8)	nd	nd
10 Jun 1989	69.1 (5.2)	36.2 (2.3)	nd	nd
16 Aug 1989	68.9 (4.2)	36.5 (1.8)	nd	nd

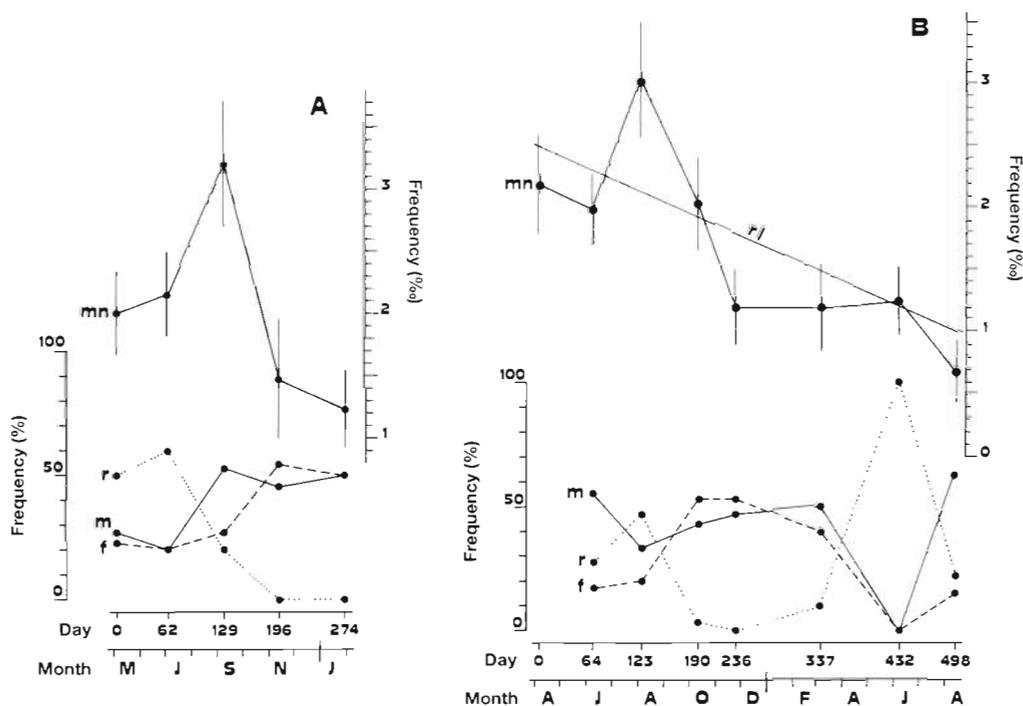


Fig. 1. *Mytilus galloprovincialis*. (A) Mussels from North Adriatic, May 1988 to February 1989. (B) Mussels from North Tyrrhenian, April 1988 to August 1989. Upper graphs: pattern of the mean frequencies (‰) of micronuclei (mn) with 95 % confidence limits of the mean; rl: regression line. Lower graphs: frequencies (%) of males (m), females (f) and mussels with regressed gonads (r). Abscissa: time in days and months

thus also as a function of the age of the animals (regression significance:  $p < 0.025$ ).

When only mussels in their second year of life are considered (i.e. when they have practically ceased their initial rapid increase in growth), we can conclude that base-line micronucleus frequencies change according to water temperature and that, for unpolluted areas of the Mediterranean, they may be assumed to be ca 2 ‰ in the thermal range 15 to 20 °C (spring), ca 3 ‰ at temperatures above 20 °C (full summer), and 1 ‰ when temperatures fall below 15 °C (winter). These values are in agreement with those reported by Migliore et al. (1989) who, at our same Tyrrhenian station, found a mean micronucleus frequency of about 2 ‰ in specimens collected at the beginning of June 1987. In contrast, these values are lower than those which we found in mussels from the same area in August 1987 (Brunetti et al. 1988; Stn A). As we ascertained by comparing results obtained from the left and the right gills of the same specimens (data not shown), this difference is due to the technique adopted in preparing cell suspensions. Our previous method, gill laceration, furnished 'dirtier' material and an over-evaluation of micronucleus frequency.

In conclusion, comparing samples collected in different areas and/or seasons, the thermal pattern of waters

and specimen age will have to be born in mind. Unfortunately, at present there is no easy, sure method of ascertaining the age of mussels, so that in environmental studies some kind of control will have to be devised by researchers.

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