

Histology, cytogenetics and cytofluorimetry in diagnosis of neoplasia in *Macoma balthica* (Bivalvia, L.) from the southern Baltic Sea

KATARZYNA SMOLARZ^{1*}, TRISTAN RENAULT² and MACIEJ WOŁOWICZ¹

¹ Laboratory of Estuarine Ecology, Institute of Oceanography, University of Gdańsk, Al. Marszałka J. Piłsudskiego 46, 81-378 Gdynia, Poland

² IFREMER, Laboratoire de Génétique et Pathologie, 17390 La Tremblade, France

Abstract — This study presents results of histopathologic, cytogenetics and flow cytometry analyses performed on *Macoma balthica* collected from the Gulf of Gdansk (Baltic Sea) in 2003 in order to compare the techniques for diagnosis of neoplasia. The proportion of affected clams gave a crude prevalence of 15.7 %. The four stages of the disease defined by histology and three stages of neoplasia defined by flow cytometry were reported. Stage I defined by flow cytometry corresponded to stages I and II defined by histology. Chromosome analysis did not lead to a staging of neoplastic progression.

Both cytogenetics and flow cytometry indicated a difference in the DNA content of non-neoplastic and neoplastic cells. Cytogenetics examination marked that the range of chromosome numbers scored in abnormal mitosis corresponded to pentaploid-like cells (2.37 x diploid) and was similar to the mean DNA quantity identified using flow cytometry (2.36 x diploid). These methods generally have lower diagnosis sensitivity because with both techniques only a part of an animal can be studied. Thus, histology examination appeared to be the most sensitive tool for detection of the possible foci of neoplastic cells, metastasis and rare tumour cells freely circulating in the hemolymph in the early stages of the disease. Cytogenetic analysis has been considered as an important tool for the evaluation of aquatic environment quality as well as for the ecological risk assessment. Flow cytometry provided a rapid and easy method for discrimination of the aneuploid cells within thousands of cells per individual. Thus, in diagnosis of early stages of the cancer as well as early metastasis histology analyses should be performed. Chromosomes analysis and flow cytometry examination are important techniques for detection abnormalities in cell division, cell viability and DNA quantity. They appear to be very important in diagnosis of tumors based on high aneuploidy level.

Key words: Baltic Sea, cytofluorimetry, cytogenetics, histology, *Macoma balthica*, neoplasia

INTRODUCTION

FARLEY (1969) for the first time reported the occurrence of proliferative disorders in *Crassostrea virginica*, *C. gigas* and *Mytilus* sp. characterised by nuclear and cellular polymorphism, nuclear hyperchromatism and a high frequency of mitotic figures. Since then the disease was linked to the presence of more than one nucleoli in the nucleus, the presence of ectopic AgNORs and a high nucleus to cytoplasm ratio (e.g. ELSTON *et al.* 1992; KRISHNAKUMAR *et al.* 1999; SMOLARZ *et al.* 2003; MIX 1983). Moreover, similar disorders have been described in more than 20 bivalve spe-

cies around the world (ELSTON *et al.* 1992; PETERS *et al.* 1994; ALONSO *et al.* 2001; VILLALBA *et al.* 2001) including *Macoma balthica* (L.) from the Baltic Sea (PEKKARINEN 1993). In *M. balthica* from the Gulf of Gdansk neoplasia was for the first time identified by the occurrence of abnormal metaphases in the respiratory system and was reported by THIRIOT-QUIÉVREUX & WOŁOWICZ (1996). To date similar disorder has been described in *Mya arenaria* collected from the same region (WOŁOWICZ *et al.* 2000).

From 1969 neoplasia has been diagnosed in bivalves using a number of methods. Virtually all bivalve cancers were discovered by histology examination with the light microscope. This technique also appears to be the most sensitive tool to diagnose early stages of the disease and possible metastasis. Assessments of cell morphology by histology or hemocytology have been reported by sev-

* Corresponding author: email: smok@sat.ocean.univ.gda.pl.

eral authors, e.g. CHRISTENSEN *et al.* (1974); MIX (1983); BERT *et al.* (1993); PEKKARINEN (1993); VILLALBA *et al.* (1997) and KRISHNAKUMAR *et al.* (1999). Specific antibody staining has been used by e.g. SMOLOWITZ & REINISCH (1986); MIOSKY *et al.* (1989). Chromosome analysis has also been used by RENO *et al.* 1994; THIRIOT- QUIÉVREUX & WOŁOWICZ (1996; 2001) and SMOLARZ *et al.* (2003) and provides data about chromosomal disturbances that might reflect a rapid response of organisms to environmental pollutants (DEPLEDGE 1997). Techniques in genetics have been used by e.g. GEE *et al.* (1994) and HOUSE *et al.* (1998). DNA measurement using flow cytometry, based on different fluorescent markers and differences in DNA quantity between non- neoplastic and neoplastic cells, generates data on phenotyping (size, complexity etc.), ploidy level and cell function (ROMBOUT *et al.* 1996; RENAULT *et al.* 2001; GAGNAIRE *et al.* 2003). As such, it provides a tool to monitor cell viability and cell activities (BROUSSEAU *et al.* 2000; CARDENAS *et al.* 2000; XUE *et al.* 2001) and has been used by e.g. ELSTON *et al.* 1990; MOORE *et al.* 1991; RENO *et al.* 1994.

In our study three different techniques have been used in order to compare results and to obtain the most suitable and sensitive tool for neoplasia detection in clams from the Gulf of Gdansk. All individual samples were analysed in parallel using histology, cytogenetics and flow cytometry.

MATERIALS AND METHODS

Macoma balthica L. (16 ± 4 mm mean valve length) were collected by dredging in the Gulf of Gdańsk (Baltic Sea) at areas marked on Fig. 1. Clams were maintained for a few days in laboratory condition (salinity 7-8 psu, $13 \text{ C}^\circ \pm 2 \text{ C}^\circ$) and fed daily with *Isochrysis sp.* algae. Previous to sampling and analysis, the animals were incubated for 8 h in colchicine (antimitotic agent, 0.005 % in sea water, 7 psu). All animals were prepared to perform histology, chromosomal analysis and flow cytometry, as described below.

Histology - The soft tissue of each individual was placed in Davidson's fixative (35% sea water, 35% 95° ethanol, 3% formaldehyde, 12% glycerol and 9% glacial acetic acid) for 48 hours. Samples were dehydrated through ethanol series, cleared in xylene and embedded in paraffin. Histological blocks were sectioned at 2 μm thickness. Sections were stained with hematoxylin and eosin (H & E) and then examined with light microscopy. Identified cases of neoplasia were classified from I to IV (CHRISTENSEN *et al.* 1974; PEKKARINEN 1993):

I) Neoplastic changes confined to gill epithelia, characterized by nuclear and nucleolar enlargement and multiple, occurrence of abnormal cell division;

II) Locally invasive lesions invading underlying tissues and large number of abnormal mitosis;

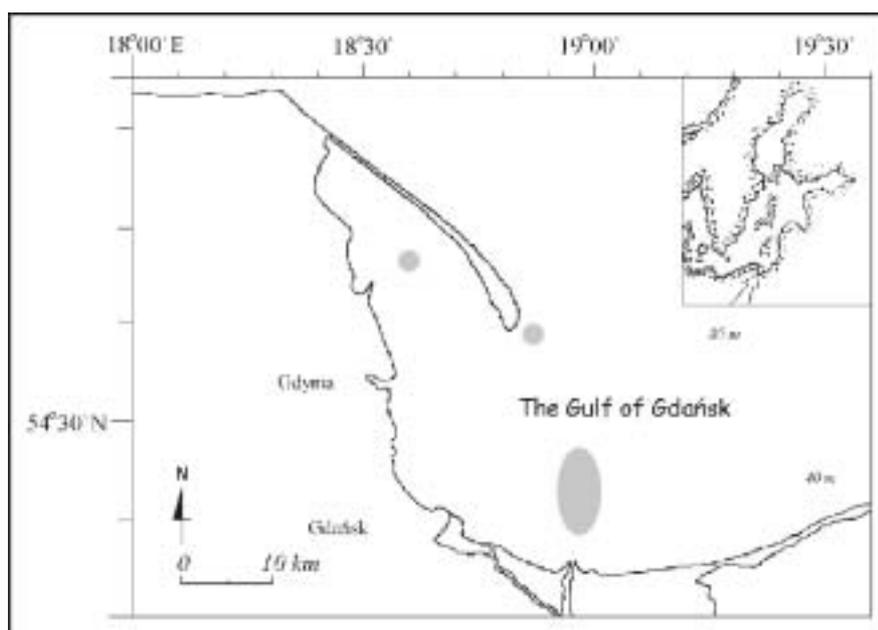


Fig. 1 — The sampling area in the Gulf of Gdańsk (Baltic Sea, Poland).

III) Appearance of neoplastic cells in other tissues;

IV) Widespread proliferation of neoplastic lesions.

Chromosome analysis - A final portion of the gill from each animal was placed in sodium citrate (0.9 %) in distilled water for 45 min, then fixed in a solution of absolute ethanol: acetic acid (3:1) (2 x 20 min and 2 x 10 min). Slide preparation was made with tissue from each individual using an air-drying technique (THIRIOT-QUIÉVREUX & AYRAUD 1982). Slides were stained for 8 min with Giemsa (Sigma, 4 %, pH 6.8). The scoring of mitosis under light microscopy was carried out to confirm the presence of neoplasia in gills.

Flow cytometry - The preparation of cell suspension for flow cytometry analyses was made using the technique previously developed by SMOLARZ *et al.* 2005. A portion of gill tissue from each individual was placed in 0.5 ml sterile distilled water, grounded in an Eppendorf tube, filtered through a 60 µm sterile mesh, adjusted to 0.5 ml with distilled water, left for one hour on ice and then analysed by flow cytometry.

The cell cycle was assessed by staining DNA with propidium iodide (PI, Interchim) (50 ng.ml⁻¹, 30 min in the dark and room temperature). Gill cells were analysed with a flow cytometer (EPICS XL4, Beckman Coulter). PI red was collected through a 600 nm band pass filter. For each gill sample 3000 events were counted.

Two parameters were used to define ploidy in *M. balthica* populations: the percentage of cells in peaks B and C and the ratio between the mean fluorescence values of all peaks (B\B+C). Three stages of neoplasia were separated, taking into account the importance of the peak C. Stage I was characterised by more than 10 % of cells in the C peak and mean fluorescence ratio around 0.8. Stage II consisted of more than 25 % of cells in the C peak and a 0.7 mean of fluorescence ratio. Stage III of the neoplasia was characterized by more than 40 % of cells in the C peak and a mean B/B+C ratio around 0.5.

RESULTS

On the basis of histology, cytogenetics and flow cytometry the prevalence of neoplasia in *Macoma balthica* from the Gulf of Gdańsk was studied. A total of 300 clams were analysed. 47 clams were identified as having neoplasia that led to a crude prevalence of 15.7 %.

Histological representations of non-neoplastic gills and gills with neoplasia are demonstrated in Figures 2a – c. Affected cells were large, actively proliferative with pleomorphic nuclei freely circulating in the hemolymph (Fig. 2c). In later stages

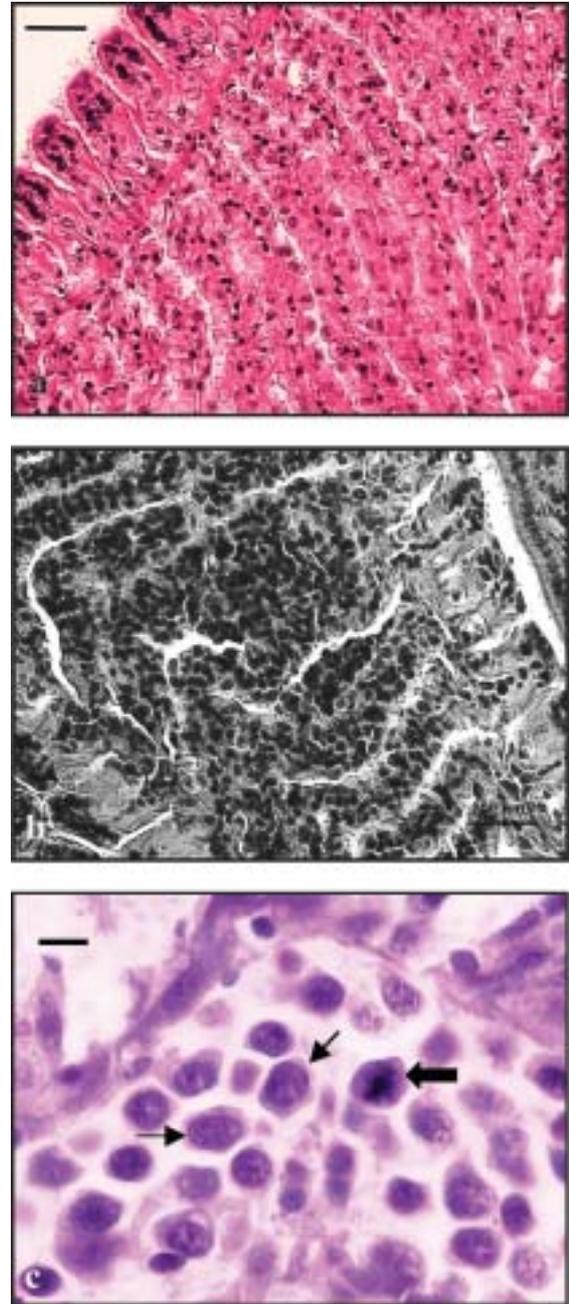


Fig. 2 — Histological section through *Macoma balthica* without neoplasia showing (a) foci in interlamellar septum of gills and gill filaments, and with neoplasia showing (b) stage IV of the disease manifested by severely damage gills, scale 100 µm, H&E (c) large neoplastic cells, actively proliferative with pleomorphic nuclei (arrows) and mitotic figures (block arrow), scale 5 µm, H&E.

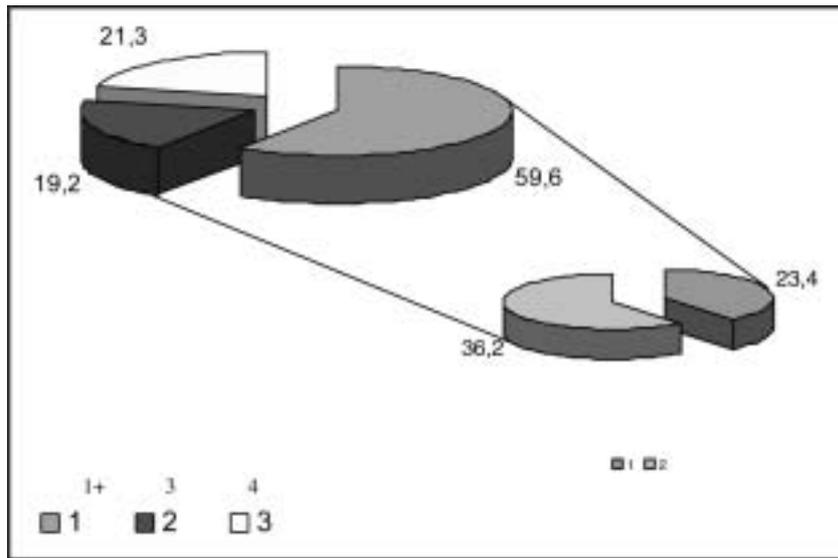


Fig. 3 — Average contribution of affected *M. balthica* defined using histology (four stages of the disease) and flow cytometry (three stages of the disease) in the Gulf of Gdansk. Stages I and II defined by histology correspond to the stage I defined by flow cytometry. Data given in [%].

of the disease affected cells often invaded surrounding organs and diffused through the connective tissue into the body wall. Four stages of the disease were clearly separated in clams with neoplasia. The specific prevalence of stages I to IV ranged from 19.2 % to 36.2 %. 11 animals were classified as stage I, 17 as stage II, 9 individuals as stage III and 10 clams as stage IV of the disease. Figure 3 presents the comparison between staging of neoplasia diagnosed by histology and flow cytometry.

In animals with neoplastic features the chromosomal number ranged from 59 to 105 with a median of 90 chromosomes (Fig. 4). This range corresponds to 1.55-2.76 (median 2.37) of diploid. The mitotic activity increased with the disease severity, moreover chromosome analysis showed a higher presence of abnormal mitosis in all clams (up to 500 mitoses, data not shown). In all abnormal metaphases, telocentric and microchromosomes were present. Additionally, abnormal cells were characterised by a higher quantity of genetic material. Chromosome analysis did not lead to a staging of neoplastic progression.

The cytometric profile of highly affected clams *M. balthica* with neoplasia is shown in Figure 5. Depending on individuals and the stage of the disease variations in the prevalence of normal and abnormal cells, shown as fluorescence peaks, namely B and C were observed. Three stages of neoplasia were clearly separated ($p \leq 0.0001$, ANOVA). For stages I to III the specific preva-

lence ranged from 19.2 % to 59.6 %. The presence of 28 animals in the stage I of neoplasia was noted, 9 individuals in the stage II and 10 clams in the stage III of the disease were observed.

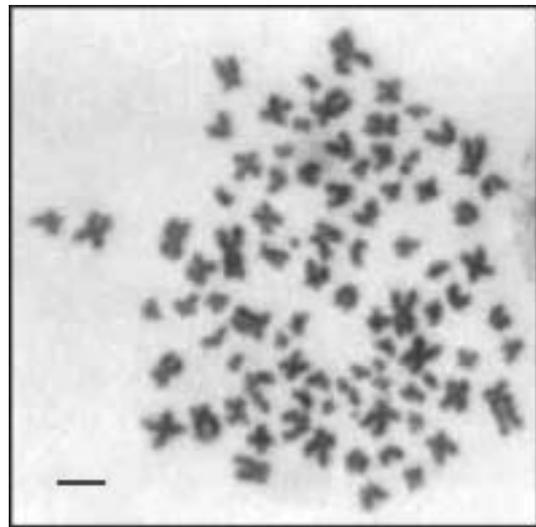


Fig. 4 — Giemsa-stained metaphase of affected *M. balthica* with 87 chromosomes, see micro- and telocentric chromosomes, scale bar 5 μ m.

DISCUSSION

The occurrence of an arbitrary proportion of neoplastic cells relative to normal cells has become a widely accepted basis for diagnosis and

staging of bivalve disseminated neoplasias. As the discrimination of neoplasia from atypical but non-neoplastic cells is very difficult, reliable diagnosis of the tumour was a challenging task, especially in early stages of the disease. Thus, all diagnosis methods are characterised by a lower limit of detection, and their obvious limitation is that in many cases the sacrifice of an animal is required (ELSTON *et al.* 1992).

Histology, cytogenetics and flow cytometry techniques used in our study provide excellent morphological details and amplification of the behavioural differences of normal and neoplastic cells. The four stages of neoplasia defined by histology (CHRISTENSEN *et al.* 1974; PEKKARINEN 1993) and three stages of the disease defined by flow cytometry were reported in animals collected from the Gulf of Gdansk. Stage I defined by flow cytometry corresponded to stages I and II defined by histology. Chromosome analysis did not lead to a staging of neoplastic progression.

Virtually all bivalve neoplasias were discovered by histology examination with the light microscope (e.g. FARLEY *et al.* 1969; FARLEY 1976 a and b; RASMUSSEN 1986; BERT *et al.* 1993; VILLALBA *et al.* 1995; VILLALBA *et al.* 1997; KRISHNAKUMAR *et al.* 1999; ALONSO *et al.* 2001; VILLALBA *et al.* 2001; DUNGAN *et al.* 2002). In our study affected clams displayed the same kind of pathological abnormalities: hypertrophied cells with enlarged nuclei and multiple nucleoli and invasion of neoplastic cells in the later stages of the disease as previously described by e.g. CHRISTENSEN *et al.* (1974); ELSTON *et al.* (1992); PEKKARINEN (1993); PETERS *et al.* (1994). Heavily affected clams showed infiltration of neoplastic cells in all tis-

sues. Abnormal cells were present in the connective tissue of gills at the first stage of neoplasia, which may correspond to a hemocytic origin of the disease, as previously described in other bivalve species (BOWER *et al.* 1994; RODRIGUEZ *et al.* 1997). MOORE *et al.* (1991) reported that histology was the most sensitive tool according to diagnosis of early stages of neoplasia. Also in recent work histology examination appeared to be the most sensitive method to detect possible foci of neoplastic cells, metastasis and rare tumour cells freely circulating in the hemolymph in the early stages of the disease. However, also this method may miss focal concentrations of abnormal cells, unless serial sectioning of entire animals is performed.

Chromosomes analysis indicated that the diploid chromosome number for *M. balthica* is 38, while abnormal metaphases mainly consisted of around 90 chromosomes (THIRIOT-QUIÉVREUX & WOŁOWICZ 1996; 2001). A high mitotic index was also observed in clams with neoplasia. Cytogenetics examination marked that the range of chromosome numbers scored in abnormal mitosis corresponded to pentaploid-like cells (2.37 of diploid) and was similar to the mean DNA quantity carried out using flow cytometry (2.36 of diploid). DEPLEDGE (1997) pointed on that chromosomal disturbances usually reflect a rapid response of organisms to environmental toxicant exposure and can provide early warning signs of adverse long-term effects in the population of aquatic biota. Cytogenetic analysis has been considered as an important tool for evaluating the quality of aquatic environments as well as for the ecological risk assessment. Cytogenetic damage in aquatic

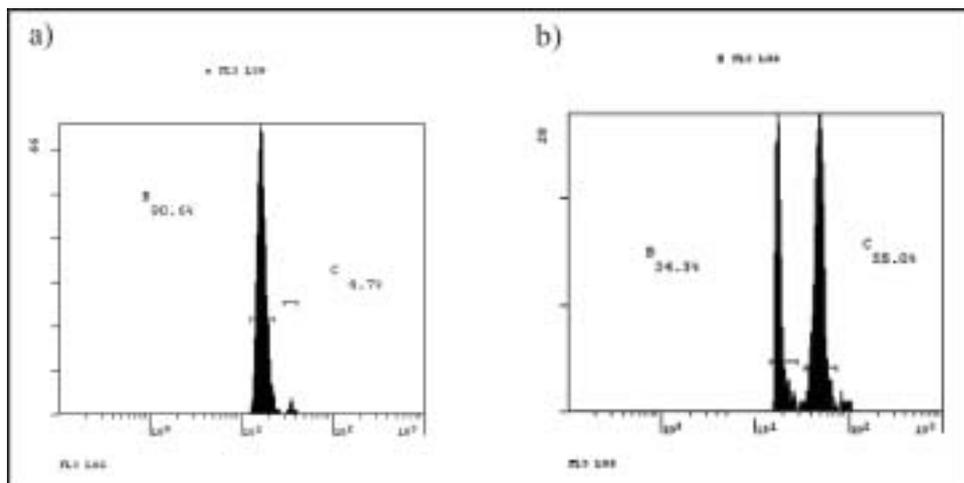


Fig. 5 — Fluorescence histogram of (a) normal cells of baltic clams and (b) affected with neoplasia *M. balthica*, cytometric profile of stage III of the disease.

organisms has also been considered as a biomarker of environmental exposure in polluted areas (e.g. BARŠIENĖ & BARSYTE LOVEJOY 2000; BARŠIENĖ 2002). Moreover, the data currently available in literature demonstrate that cytogenetics markers such as nuclear organizer regions (Ag-NORs) detected in chromosomes may be an independent prognostic factor in several types of malignant human tumours (e.g. PICH *et al.* 1995; 2000; 2002) and can be implied as a prognostic function for bivalves tumours (SMOLARZ *et al.* 2003). Both, cytogenetics and flow cytometry indicated that there was a difference in the DNA content between non-affected and neoplastic cells characterised by a high aneuploidy rate, cell viability and cell cycle phase.

LOWE & MOORE (1978), studying *Mytilus edulis* from England, for the first time measured DNA content of neoplastic cells relative to normal hemocytes. ELSTON *et al.* (1990) have introduced this technique for measuring DNA content to study bivalve neoplasias. The diagnosis of tumour was based on measuring the ploidy level due to differences in DNA content and quantity between non-neoplastic and neoplastic cells. Low prevalence of dividing cells corresponded to the normal status of the bivalve while a high number of aneuploid cells were characteristic for cancer cells population. ELSTON *et al.* (1990) and MOORE *et al.* (1991) found out that systemic neoplasia in *Mytilus* sp. exists in two major forms characterized by the occurrence of tetraploid- and pentaploid-like cells. The existence of these alternative forms of the cancer was not confirmed by RENO *et al.* (1994) for *Mya arenaria*. In our study pentaploid-like cells were predominant in gills. Thus, flow cytometry appeared to be the only reliable tool for performing a DNA quantification of cancer cells. This technique also provided a rapid method for neoplasia detection and discrimination of aneuploid cells as well as for identification of the ploidy of the population. However, both chromosome analysis as well as flow cytometry generally effects with lower sensitivity for diagnosis. RENO *et al.* (1994) found out that circulating cells of all clams with neoplasia greater than 10 % examined by flow cytometry showed distinct DNA aneuploidy.

Tumour pathology connected to *Macoma balthica* from the Gulf of Gdańsk appears to be most likely attributed to indirect effects of pollution. Because of serious pollution, asymmetry in contamination and low biodiversity, this ecosystem provides an ideal environment to test cause-and-effect relationships between pollutants and

their biological effects. Thus, studies elucidating the environmental significance of bivalve disseminated neoplasia and assessing its biological risk at the population and ecosystem levels are necessary to continue.

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