Methods for Assessing the Effects of Chemicals on Reproductive Functions
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Methods for Assessing the Effects of Chemicals on Reproductive Function in Marine Molluscs

D.R.Dixon

1 INTRODUCTION

Marine molluscs, and in particular those from estuarine and coastal environments, are being increasingly exposed to a wide variety of chemical pollutants resulting from man's activities (i.e. oils and detergents, heavy metals, and halogenated hydrocarbons). In addition to anthropogenic pollution, these organisms also come in contact with chemical substances with biogenic (e.g. algal exudates) and terragenic/lithogenic (e.g. land run-off) origins (Roberts, 1976).

It is now recognized that a number of marine mollusc species have the ability to accumulate toxic chemical substances in their tissues, at levels often far exceeding those present in the surrounding water (Goldberg, 1978). Several of these contaminants are known to inflict damage on the tissues, with the consequence that the life processes of these organisms may become seriously impaired (e.g. Bayne *et al.*, 1979). Such effects finally become realized at the population level as a result of reproductive dysfunction, through reduced fecundity, impaired gamete quality and reduced larval viability (e.g. Bayne, 1972, 1976; Bayne *et al.*, 1975, 1978; Sastry, 1979).

This paper refers, for the most part, to methods relating to the reproductive stages in the life cycle of the bivalve, *Mytilus edulis* L. This has been fully intentional since the blue mussel, and other closely related species, occupies a central position in marine environmental toxicology. As a group, marine mussels are very important to many aspects of the ecology of coastal waters, notably to the productivity of the shallow-water benthos and to aquaculture. This importance is underlined by the adoption of 'marine mussels' as a theme and as subject organisms in several international, pollution-related, biological programmes (e.g. International Biological Programme, see Bayne, 1976; 'mussel watch', see Goldberg, 1978).

Figure 19.1 shows stages in the reproductive cycle of *Mytilus edulis* referred to later in the text. These reproductive stages have been identified for detailed methodological discussion:

(1) gonad (mantle tissues)—stereology;
(2) gametes, embryos and larvae (trochophore and prodissoconch I)—acute toxicity;
(3) gametes and embryos—cytogenetics.

Description is also included of a new and promising test organism for field and laboratory studies of induced embryo abnormalities, the rough periwinkle, *Littorina saxatilis*, which appears for the first time here.

No attempt has been made to give a comprehensive coverage of all methods and approaches that are recorded in the molluscan literature, and which could be applied when assessing the effects of chemicals on reproductive function in the molluscs. It will be noticed that energetic considerations have been omitted from this paper, as have the interesting concept of maternal contributed RNA and its role in early protein synthesis in the larva (e.g. Bayne, 1972; Kidder, 1976; McLean, 1976). Further details of the energetic approach can be found in Bayne (1976) and Bayne *et al.* (1975, 1978, 1979).

2 GONAD CONDITION INDEX

In *Mytilus*, gametes are produced in transitory follicles located in the mantle which lines the shell valves (Figure 1). There is a well-defined annual cycle relating to the reproductive processes in *Mytilus*, with a marked alternation between the periods of nutrient accumulation and gamete production (e.g. Lubet, 1957; Seed, 1975; Lowe *et al.*, 1982). The timing and pattern of gamete release is dependent upon climatic and geographic factors (Bayne, 1976); in Britain, populations living in the north spawn only once a year (late spring), whereas those in the south west, an area of milder winters and warmer summers, may spawn twice, once in spring and again in late summer (Seed, 1976).

This gametogenic rhythm is susceptible to disturbance from a number of physical and chemical factors in the environment. Recent studies by Lowe in this laboratory have demonstrated quantitative techniques for assessing the gametogenic cycle and its disruption by environmental disturbance. What follows is taken in large part from Lowe *et al.* (1982). Both natural (e.g. climatic) and anthropogenic (chemical pollution) stressors have been shown in the laboratory, and under field conditions, to produce quantitative changes in mantle condition in exposed populations of mussels. By measuring, with *stereological techniques*, the relative volume fractions of the vesicular cells (VC), adipogranular cells (ADG) (these two cell types comprising the nutrient store of the mantle) and the developing and ripe gametes, Lowe has been able to derive precise information relating to the reproductive status of mussels from polluted and unpolluted populations, or, by repeated sampling, obtain a quantified expression of the changes in mantle condition attributable to their exposure.

Preliminary investigations showed that the distribution of reproductive

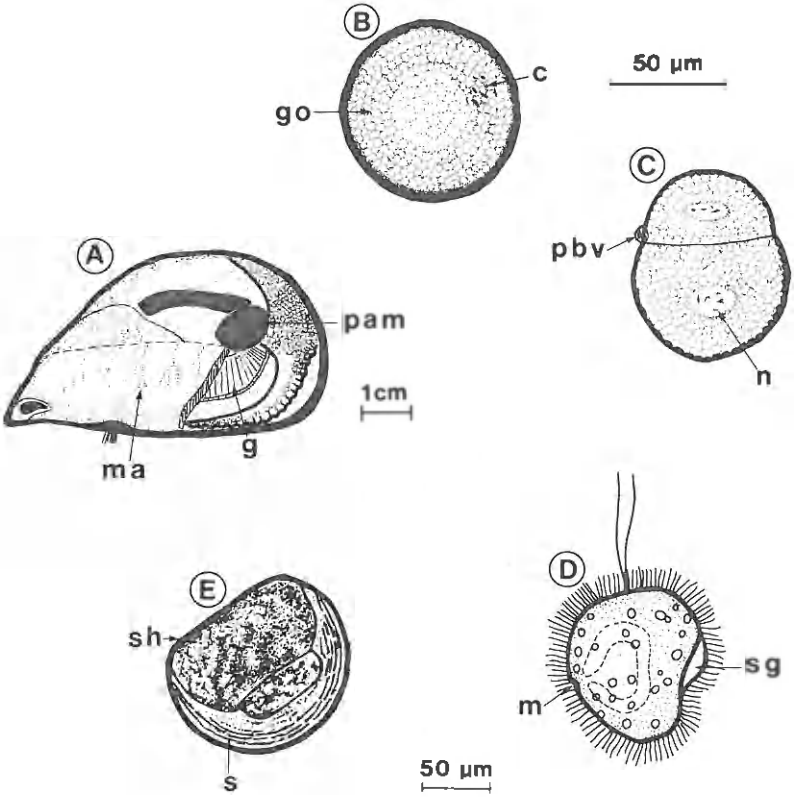

Figure 1 Life-cycle stages of *Mytilus edulis*, referred to in the text. A, adult mussel with one shell valve removed to display the internal organs; B, egg; C, 2-cell embryo showing asymmetrical first cleavage; D, trochophore larva at 24 hours (drawn from Sastry, 1979); E, prodissoconch I-stage (first shell) larva at 48 hours; c, chromosomes; g, gill; go, granular ooplasm; m, mouth; ma, mantle; n, nucleus; pam, posterior adductor muscle; pbv, polar body vesicle; s, shell; sg, shell gland; sh, straight hinge

follicles throughout the whole of the mantle is not subject to planorelated variation, i.e. are for all practical purposes distributed at random with respect to mantle orientation. Consequently, a reasonable estimation of the overall mantle condition can be derived simply from examination of a thin section taken in any one plane. In our laboratory, tissue sections at 5 μm are examined with a Zeiss (Jena) compound microscope fitted with a Weibel eyepiece graticule (Graticules Ltd) at a magnification of × 240. Point counts (Weibel and Elias, 1967) on ADG cells, VC cells, developing and morphologically ripe gametes, and areas of empty follicles resulting from spawning activity are made on five fields per animal to quantify the volume fraction of the different tissue components.

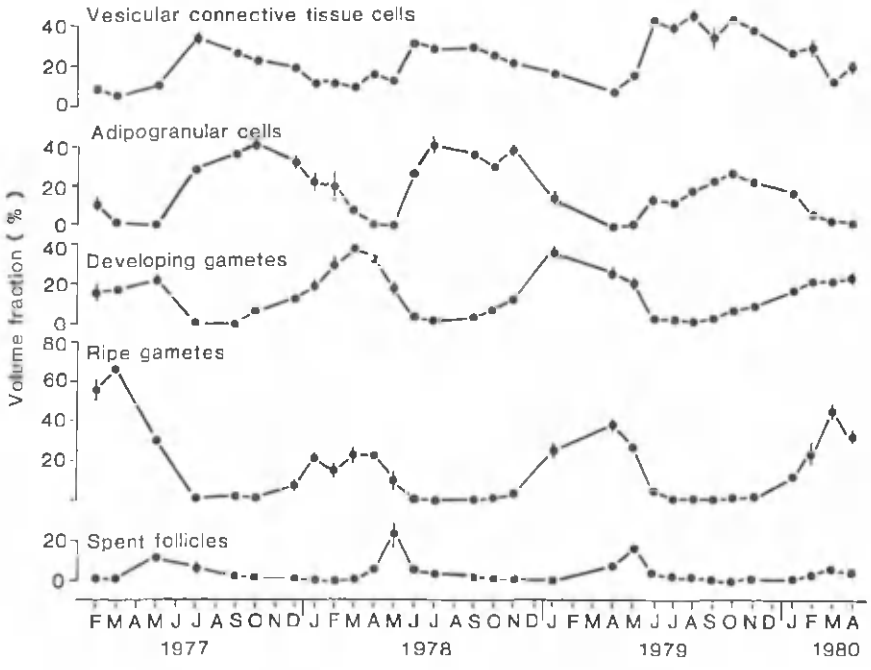

Figure 2 Volume fractions (means ± 1 s.e.) of mantle tissue components from Tal-y-foel (Menai straits, N. Wales) mussels. Only standard errors which are greater than 2% are shown in the figure. Each point is based on five separate determinations

An example of the results of such a study is shown in Figure 19.2, taken from Lowe *et al.* (1982). The factors controlling the seasonal cycle of the various cell types are complex but the cycle is susceptible to alteration by pollution and preliminary evidence suggests that quantification by stereology will provide a powerful technique for determining reproductive disturbance in this and in other species.

3 ACUTE TOXICITY TESTS WITH LARVAL BIVALVES

The larvae of a number of marine bivalve mollusca (*Crassostrea virginica* Gmelin, *Crassostrea gigas* Thunberg, *Mytilus edulis* L.) have been incorporated into standardized, short-term, acute toxicity test procedures for use in evaluating pure compounds singly or in mixtures. They can also be used to measure the acute toxicity of marine and estuarine waters, and the acceptability of salt waters for culturing and testing fish and macroinvertebrates (ASTM, 1980). The stages identified for study of toxic effects are fertilized egg, trochophore larva, and 48-hours old prodissoconch I larva ('straight-hinge' or 'D-shaped' stage) (Figure 1). The following description applies for all four species identified above.

Embryos and larvae of other planktonic-spawning bivalves (Sastry, 1979) may be used in place of these, but modifications of the adult conditioning, spawning and testing regimens frequently are required (e.g. Cain, 1973; Rhodes *et al.*, 1975).

Adult bivalves possessing developing gonads are brought to the laboratory, cleaned of all fouling organisms and detritus, and to ensure complete maturation of their gametes are held (conditioned) in circulating seawater tanks at specified temperatures several degrees above the field temperature when the adults were collected. A balanced diet is essential during this period (e.g. Bayne, 1965). Spawning is induced with one or more selected physical (e.g. temperature), chemical (e.g. KCl), or biological stimuli, as discussed later. Selected densities ($< 35{,}000/1$) of the embryos are exposed to the toxicant for 48 hours, during which the embryos normally will develop into fully shelled larvae (prodissoconch I stage).

Toxicity to the prodissoconch I stage is measured as the 48-hour median effective concentration (EC_{50}) based on abnormal shell development and the 48-hour median lethal concentration (LC_{50}). Other responses may include decreased fertilization, and decreases in the rates of development of specified stages (e.g. trochophore, veliger, prodissoconch I larva).

The results describe the responses of the larvae to short-term exposures to toxicant(s) under a set of environmental conditions. Temperature, pH, salinity, suspended solids, and organic metabolites from phytoplankton and bacteria are some of the water quality characteristics that may influence the effect of the toxicant by stressing the developing embryos or larvae or by altering the physicochemical form, availability, or concentration of the toxicant. Specific guidelines are laid down regarding test procedures in standard E 724–80, published by the American Society for Testing and Materials (ASTM, 1980).

4 CHROMOSOMAL ABERRATIONS AND RELATED EFFECTS

The embryos of *Mytilus edulis* are excellent material for cytogenetic analysis, and have featured in a number of studies of a cytotaxonomic nature (e.g. Menzel, 1968; Ahmed and Sparks, 1970; Ieyama and Inaba, 1974). More recently, both indirect and direct evidence has been presented for induced chromosomal aberrations in the cells of adult mussels and their embroys, exposed to chronic levels of chemical pollution (aromatic hydrocarbons) under other wise natural conditions (Lowe and Moore, 1978; Dixon, 1982). These results, coupled with information from chemical investigations conducted in the laboratory (Dixon and Clarke, 1982), indicate that the chromosomes of *Mytilus* (and other marine molluscs) are sensitive to damage inflicted by certain classes of chemical pollutants in the marine environment.

The chemical effects of pollution impinging on the genetic material of marine

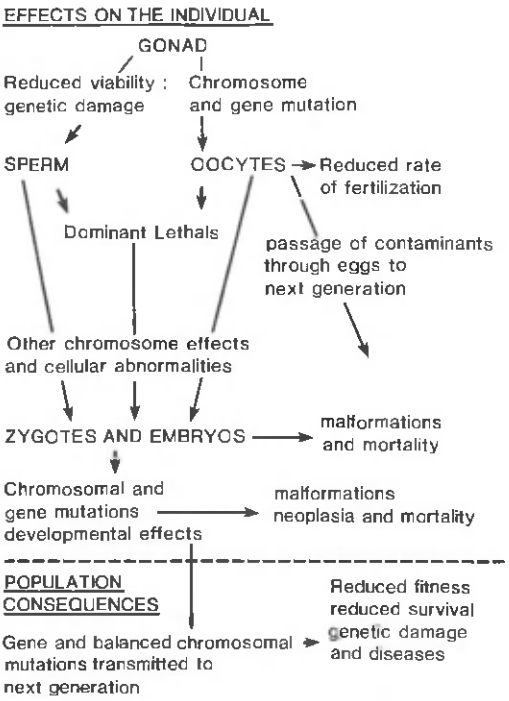

Figure 19.3 Genetic and related effects caused by toxicants impinging on the sensitive reproductive stages of a marine invertebrate. The diagram shows how the effect may be realized, as chromosome damage or otherwise, at the same stage, a closely related stage or much later on, even into the next generation

invertebrates may not always be expressed at the same stage in development as the one in which the damage was incurred. Instead, the consequences to the carrier cell, or individual may not be fully realized until some much later stage in development, even into the next generation (Figure 3).

Apart from strictly genetic effects, of a chromosomal nature or otherwise (i.e. gene mutations), there are some other types of damage resulting from pollution exposure (Table 1). When associated with the germ cells, dose–response relationships may be described in terms of fertilization rate or rates of embryogenesis. Fertilization rate measurements can be applied to both laboratory and field studies to assess either the viability of the gametes in water containing a named toxicant at a defined dose, or the condition of sperms and/or eggs originating from mussel populations exposed to pollution.

Sperm head abnormalities show a high correlation with mutagenicity for a range of chemical substances, including aromatic hydrocarbons, and could be

Table 1 The different reproductive stages of *Mytilus edulis* which can be used in testing for gentically harmful agents in seawater

Material exposed to toxicant	Maximum exposure time	Stage at which scored	Observed effects
Gonad (adult mussel)	Months	Sperms	Malformations of head
		Oocytes	Chromosome damage, e.g. translocation heterozygotes, aneuploids and polyploids
Gametes	1.5 hours (s.); 6 hour (oo.)	Fertilization	Reduced rate
Fertilized oocytes	1 hour	Same or early cleavage	Chromosomal and cellular abnormalities, e.g. abnormal polar body vesicle, septate ooplasm
Early stage embryos (< 8 cells)	7 hours	Same	Chromosomal aberrations, i.e. numerical and structural abnormalities; developmental effects, e.g. premature loss of synchrony, malformations

s., sperms; oo., oocytes

applied routinely to field monitoring of pollution-stressed mussel populations. Since this effect has its origin at some stage during spermatogenesis (as yet undefined), laboratory screening of chemicals with this technique, would require the maintenance of groups of mussels, collected from clean sites, under conditions of temperature and feeding suitable for gametogenesis to take place (Bayne, 1965; Hrs-Brenko, 1973), while in the presence of controlled levels of toxicant. Effects on synchrony of development and the different types of developmental abnormalities arising from contact with polluting substances can similarly be applied to both laboratory and field studies. Table 19.1 shows the various reproductive stages of *Mytilus* (or other similar mollusc) which have been identified for the investigation of chemical effects, the types of effects observed at any particular stage, and the range of exposure times it is possible to achieve by selecting different reproductive tissues. It is evident that the system has considerable flexibility and consequently is capable of being adapted to suit the particular requirements of the investigator; recognizing the limitations imposed only by the biological material itself.

4.1 Spawning

A large literature has grown up around this subject; for key references see reviews by Bayne (1976) and Sastry (1979). A female mussel will produce during the course of a single breeding season something in the order of 3 million eggs. These eggs are not all released at one time; spawning may be protracted, taking a period of days or even weeks, depending upon local conditions of temperature, tidal cycle, etc. In the laboratory mussels must be stimulated to spawn artificially by means of chemical or physical stimuli, or combinations of both types. The methods reported range from mechanical shaking (in a bucket), to mild electrical shocks, temperature stimuli, and various chemical techniques (for further discussion of these methods see above references).

First and foremost, it is important that the animals should be in a reproductively ripe state. Mantle squashes should reveal large numbers of mature gametes in both sexes. Ripe oocytes are large ($\simeq$ 70 μm in diameter), orange in colour, and lack any sign of the stalk which attaches the immature oocyte to the follicle wall. When first released from the mantle tissues the spermatozoa are inactive, but should become motile after a few minutes contact with sea water.

Mussels containing ripe gametes can be induced to spawn artificially by first injecting 0.5 ml of 0.5 M KCl into a large central blood sinus of the posterior adductor muscle; the shell valves should be wedged apart during this procedure. After needle and wedge are removed, the mussels should be placed beneath a 60-watt lamp for about 20 minutes until the shells have warmed through. This is followed by imersion in seawater at 20 °C, taking care to release any air trapped inside the shell when doing so. Spawning should commence within the next few hours. Using this method the author has consistently obtained 50% or greater spawning success.

To avoid early fertilization of newly spawned eggs, any males which are seen to be releasing sperms should be transferred to a separate container and the water in the holding tank changed. As a further precaution, the eggs should be pipetted into small beakers and the water changed to remove any sperms which may present. Adult mussels collected at the time of the population spawning can be kept temporarily in tanks of water at 5 °C, to prevent them from releasing gametes. Ten days before a supply of gametes is required, the mussels should be transferred, by stepwise changes (2–3 °C every 2–3 days), to water at 15 °C. It is important to feed them continuously, with a balanced algal diet (Bayne, 1976), to prevent loss of condition and concomitant resorption of gametes during this transition period.

4.2 Gamete Studies

Sperms remain viable for only 1–2 hours, depending upon the temperature, whereas eggs remain viable for up to 6 hours following their release.

Table 2 Methods for the treatment of material prior to scoring for genetic and related effects in the embryos of *Mytilus edulis*

A Spawn adult mussels and introduce sperms to eggs (i) in sea water or (ii) sea water with toxicant added. Leave to develop for 1 hour at 10°C. Centrifuge (1×10^3 revs/min/180 g for 2 min) and resuspend in sea water, repeat twice. Finally resuspend in original medium.
B Allow to develop for a further hour.
C Allow to develop for a further 2.5 hours and 6.5 hours (to allow for mitotic inhibition). Harvest embryos at the end of each period by slow centrifugation (1×10^3 revs/180g for 2 min).
D Replace medium with a dilute (0.01%) colchicine solution in seawater. After 2 hours dilute volume by 50% with distilled water and leave embryos in this hypotonic solution for 30 min.
E Fix in 3 separate changes of Carnoy's fixative (ethanol, 3 parts: glacial acetic acid, 1 part: chloroform, 1%).
F Mount in fixative and examine under medium power phase-contrast objective.
G Mount and stain in aceto-orcein. Scan under low power and examine with high-power, oil immersion lens.

Type of effect	Field collected mussels	Embryo toxicity studies
Fertilization rate	Ai, B, E, F	Aii, B, E, F
Numerical chromosome aberrations	Ai, B, D, E, G	Aii, C, D, E, G
Structural chromosomal aberrations, metaphase analysis	Ai, B, D, E, G	Aii, C, D, E, G
Structural chromosomal aberrations, anaphase analysis	Ai, B, E, G	Aii, C, E, G
Developmental abnormalities	Ai, C, E, G	Aii, C, E, G

Sperms for sperm head abnormality investigations need to be separated from the seawater by centrifugation (2×10^3 revs/min per 360g for 2 min) and resuspended in 3% ammonium formate (isotonic) to remove salt contamination. Following this treatment they should be recentrifuged and fixed with Carnoy's fixative (Table 19.2E), after which they can be mounted on a slide in a drop of fixative, the edges of the coverglass need to be sealed with glycerol–gelatin (Sigma) to reduce evaporation, and examined under high power with phase-contrast microscopy. Alternatively, smears can be prepared,at the ammonium formate stage, the smears air dried, and subsequently stained with eosin as described in the method for mammalian spermatozoa by Bryan (1970). See also Bruce *et al.* (1974).

In *Mytilus* the eggs are spawned in the germinal vesicle stage, although the germinal vesicle soon disrupts and development (of the *oocyte*) proceeds to metaphase of the first meiotic division (egg maturation), where further development is delayed until after penetration by the sperm (Longo and Anderson, 1969). It follows that if sufficient time is allowed for the completion of prophase I

by the newly spawned oocytes, in the absence of sperms, it is possible to score the metaphase plates for abnormal chromosome complements. Because of the reduced number of the units present at this stage, as a result of synapsis, it is not usually necessary to treat the oocytes in any special manner other than fix them at fertilization + 2 hours. It is possible to see both numerical (aneuploid) and structural aberrations (translocation heterozygotes) at this stage (Figure 4A)

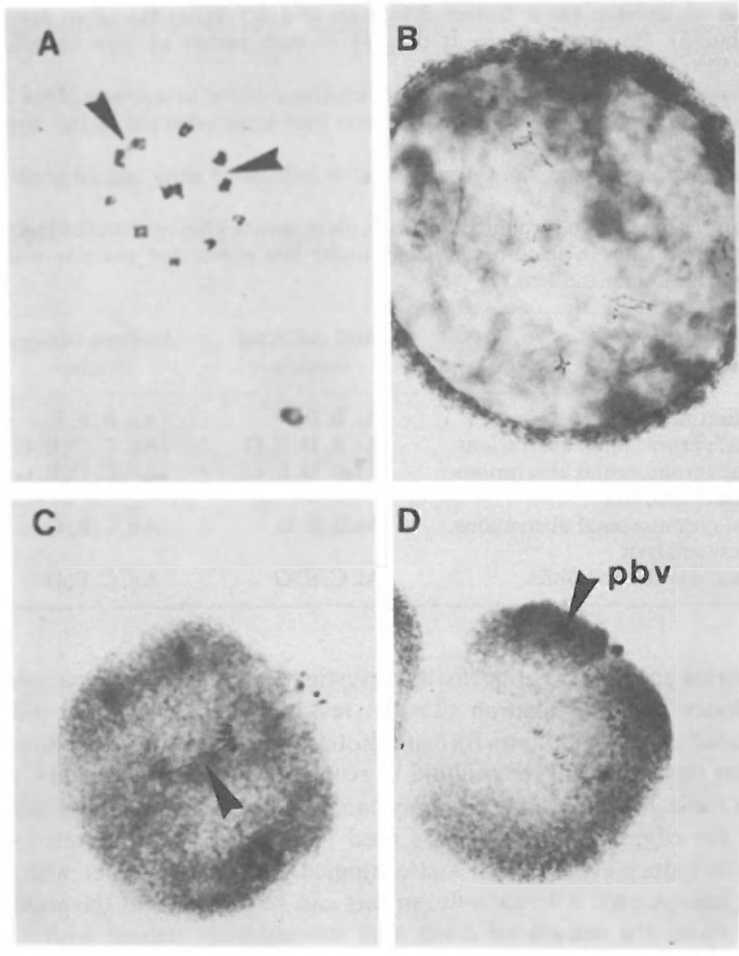

Figure 4 Normal and abnormal embryos. A, metaphase I chromosome complement with reciprocal translocation resulting in a quadrivalent configuration (arrowed); B, recently released oocyte at diplotene stage of prophase I, showing chiasmata in their primary positions; C, chromosome bridge (dicentric chromosome) linking anaphase chromatids; D, abnormal polar body vesicle (pbv), containing ooplasm, in a cell undergoing meiotic metaphase II

but the latter are better seen in freshly spawned material which is still at prophase I, (Figure 19.4B), and has yet to commence chiasma terminalization.

4.3 Fertilization Rate

Table 1 shows methods which are now being used routinely to detect chemical damage in the gametes and early-stage embryos of *Mytilus* (and other planktonic spawning marine invertebrates). Fertilization is a complex process (Monroy, 1965) involving a series of events: contact between the sperm and the egg, activation of the egg, and fusion of male and female pronuclei, which results in formation of the zygote nucleus.

Sperm penetration occurs within 5 minutes of first contact with the egg, and fusion of the male and female pronuclei after about an hour, at 20 °C. It is possible therefore to manipulate the experimental conditions to find which stage is particularly sensitive to chemical perturbation, by altering the timing and period of exposure the gametes receive. For most puposes, it is sufficient to effect fertilizations in the presence of a known concentration of toxicant and leave the eggs to develop for an hour. After this time the excess sperms are rinsed off (Table 19.2A), to prevent their remaining as contaminants on the egg surface, and the eggs left to develop for a further hour in the presence of the toxicant. By this time all fertilized eggs will have produced polar bodies, and may have commenced cleavage, and hence are distinguishable from those which are not fertilized.

With all investigations involving fertilizations, it is necessary to standardize the procedures relating to sperm and egg concentrations. It is very important not to crowd the eggs, nor to introduce too great a number of sperms (Gruffydd and Beaumont, 1970), since this can lead in decreased fertilization rate, abnormal development and polyspermy. The optimum ratio of sperms to egg is 15:1.

4.4 Chromosomal Aberrations

There are two recognized classes of chromosomal abnormalities, numerical aberrations (aneuploids, haplo/polyploids) and structural defects (achromatic gaps, chromatid deletions (open chromatid break), chromatid exchange, etc). Both classes are scorable in early stage embryos ($\leq$ 8 cells). Later stages should be avoided due to the small size of the chromosomes and the much greater risk of overlap between adjacent cells. *Mytilus* embryos have a cell-doubling time of 90 minutes at 10 °C.

Numerical aberrations may be scored in embryos from both field- and laboratory-exposed populations (Dixon, 1982), but slightly different treatments are required in each case (Table 2). Those embryos originating from samples of mussels taken from polluted field sites need to be examined when at the first, and no later than the second mitotic metaphase (alternatively the prophase I oocytes should be examined, see above) to record aberrations in their

primary state (Savage, 1975). Since the embryos have a rapid rate of cell turnover, the chromosomes do not normally achieve a state of chromatin condensation that is characteristic of more slowly growing tissues. Consequently, it is necessary to treat the cells with colchicine (Table 2D) to condense the DNA and make the individual chromosome units more easy to discern, followed by exposure to a hypotonic medium to separate them. The normal diploid number for the genus *Mytilus* is 28 chromosomes. Because planktonic spawning marine invertebrates release their young at a very early stage in their development, these organisms have not developed mechanisms for jettisoning genetically defective progeny prior to the time of their normal release (c.f. mammals). A corollary of this is that the frequency of genetically abnormal offspring amongst control groups of embryos is normally higher ($\simeq 10\%$). than is found in full-term embryos from other groups (e.g. man, Jacobs, 1972). There is good agreement however between this control value (Figure 5) and the 'primary incidence' reported for mammals (Jacobs 1972; Fraser and Maudlin, 1979).

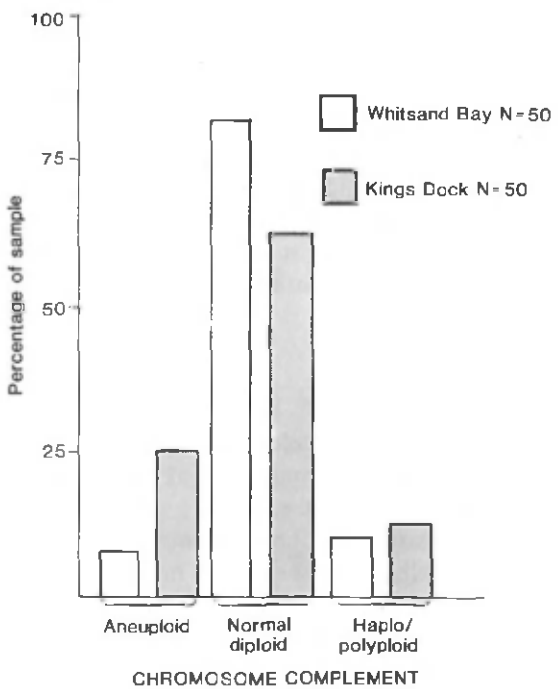

Figure 5 Percentage-frequencies of normal and abnormal chromosome complements in mussel embryos originating from polluted (King's Dock) and control (Whitsand Bay) environments

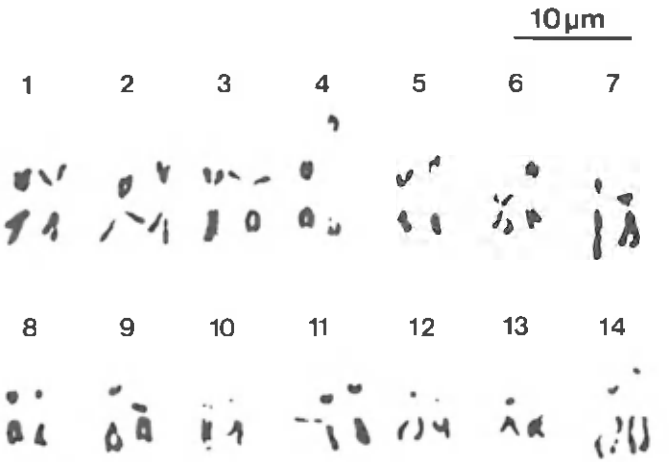

Figure 6 Karyotype for *Mytilus edulis* from King's Dock, S. Wales, based on a colchicine-treated metaphase from a 2-cell embryo. Photographed under phase contrast after staining with aceto-orcein

The normal karyotype for *Mytilus edulis* (from King's Dock, S. Wales) is shown in Figure 6, composed of colchicine-treated metaphase chromosomes from a 2-cell embryo. The chromosomes are large, homomorphic, and isopycnotic (consequently autosomal); there is evidence of balanced polymorphisms in some mussel populations (Ahmed and Sparks, 1970). The karyotype is divisible into four groups based on chromosome size (excluding centromeric distance) and position of the centromere (arm ratio): pairs 1–6 (Group A, metacentric), pairs 7 and 8 (Group B, submetacentric) pairs 9–13 (Group C, subtelocentric), and pair 14 (Group D, telocentric). With a karyotype of this type it is clearly possible to obtain detailed information relating to structural changes, and in some instances to identify the particular chromosomes affected. Methods for the treatment of laboratory and field material are given in Table 2.

It is generally recognized that metaphase analysis is particularly time consuming especially when investigating structural abnormalities, and requires a high degree of analytical skill on the part of the investigator. Detailed analysis of material collected over a few days in the laboratory may take many months of painstaking work to analyse. This has lead many cytogeneticists to opt for the simpler, anaphase method of analysis for structural chromosomal aberrations. Whilst only detailed metaphase analysis will provide comprehensive information relating to all the *visible* structural aberrations present in a cell, the reduction in sensitivity associated with anaphase analysis is outweighed by the greater numbers of cells which it is possible to analyse in a given time (Ad Hoc Committee of the EMS, 1972).

For anaphase analysis, the embryos are simply fixed, squashed and scanned for the presence of anaphase nuclei. These are then scored for chromosome bridges (dicentric chromosomes) (Figure 4C) and fragments (chromosome breaks) lying in the intercalary region separating the two groups of migrating chromatids (Nichols *et al.*, 1977).

4.5 Developmental Abnormalities

Apart from chromosomal abnormalities arising from contact of the sensitive reproductive stages of marine invertebrates with chemical pollutants in their environment, there is also a range of cellular effects which it is not possible to relate specifically to any identifiable cause, i.e. gene mutation, chromosomal aberration, or a non-nuclear event such as chemical interference to the electrochemical gradients in the cell which are at the basis of controlled growth and differentiation. It is usual therefore to categorize these effects under the general heading of *teratogenic* effects.

As is perhaps to be expected, a wide variety of different developmental abnormalities are possible, and do occur in embryos as a result of chemical interference. These range from small effects, such as the pinching off of small amounts of ooplasm into the polar body vesicle (Figure 4D) and the premature loss of synchrony in division stages, leading to uneven cell numbers, 3, 5, 7, etc., instead of the usual 2, 4, 8 etc., to major effects such as the complete schism (separation) of sister cells, with a resultant failure to produce an integrated embryo. Other effects include the formation of false septal membranes across cells without any accompanying nuclear division—this type should be watched for when measuring embryos for fertilization rate, where it is

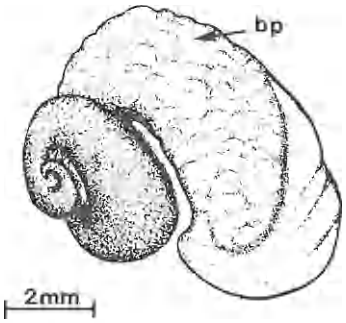

Figure 7 Female *Littorina saxatilis* removed from its shell to show the dorsally positioned brood pouch (bp) on the first body whorl

important to include only those embryos which have one or more obvious polar bodies.

I have found the gastropod *Littorina saxatilis* a convenient organism for scoring embryo abnormalities. Gastopod molluscs belonging to the *Littorina*

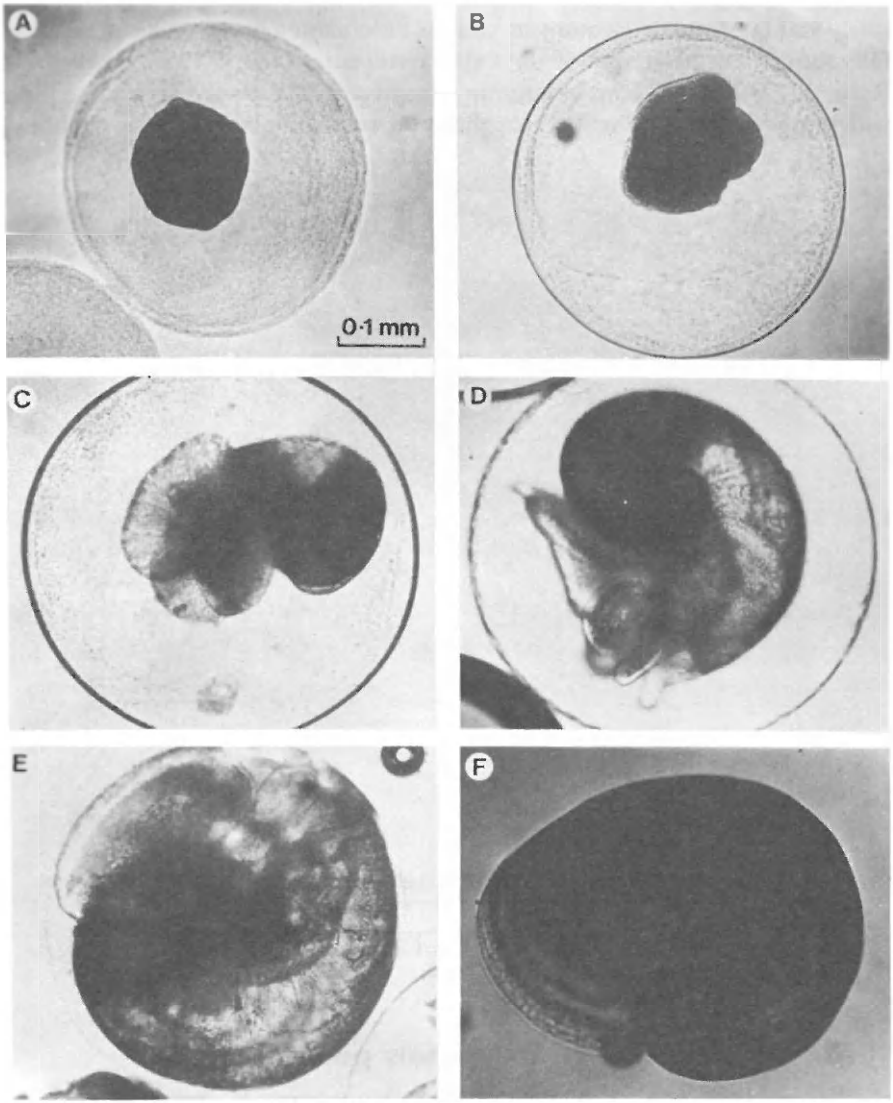

Figure 8 Normal embryonic development of *Littorina saxatilis*. A, egg (0–2 days); B, unshelled veliger (3–8 days); C, shelled veliger (9–20 days); D, late veliger (21–36 days), E, pre-emergent young (36–60 days); F, emergent stage (preserved specimen)

saxatilis 'species-complex' (Heller, 1975; Smith, 1981) retain their developing young in a specialized brood pouch, which is borne dorsally on the first body whorl of the female (Figure 7). Fertilization is internal and the fertilized eggs are passed directly from the oviduct, as batches of up to 50 in number, into the proximal region of the brood pouch, where development proceeds (Figure 8) through five, clearly recognizable stages (egg, unshelled veliger, shelled veliger (early and late), and pre-emergent young), culminating in the passive release of fully formed offspring some 60 days after fertilization (Berry, 1956; Hughes and Roberts, 1980). Adult females contain upwards of 200 embryos at any one time, with reproduction continuing throughout the year, although there is evidence of

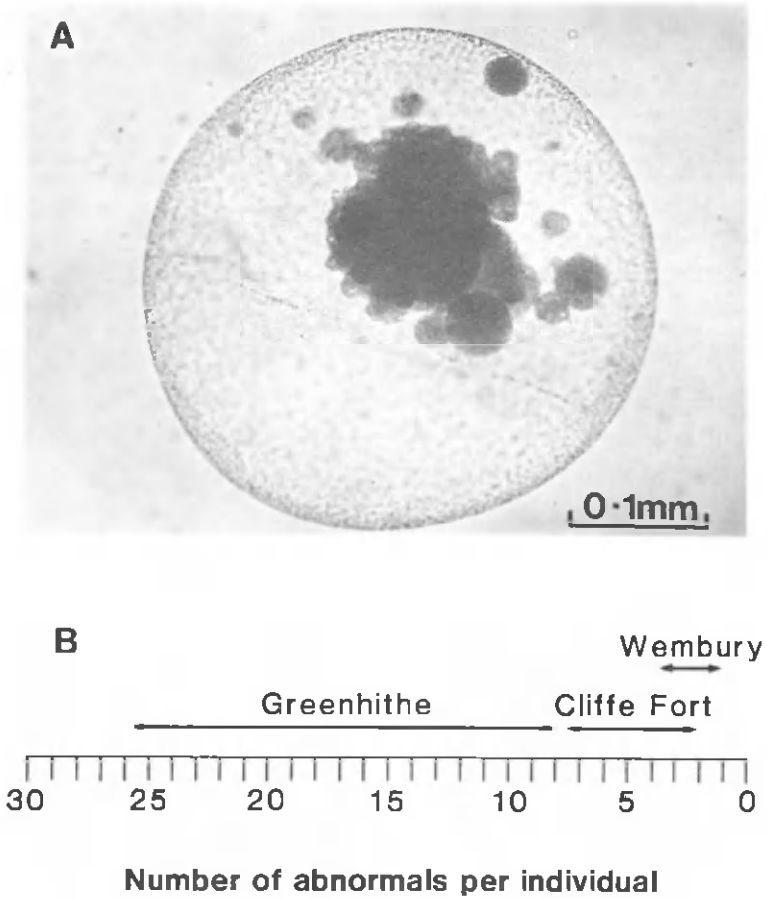

Figure 9 A, Abnormal stage II *Littorina* embryo; B, frequency of abnormal embryos ($\bar{x} \pm 2$ s.e.) in three populations (N values = 20) exposed to different levels of pollution. Greenhithe is the most polluted site and Wembury is the cleanest

a summer decline in reproductive activity in some populations. Copulating pairs are to be observed at other times of the year, on the upper shore during low tide. Ovulation occurs between 17 and 48 hours following mating (Thorson, 1946; Berry, 1956).

Littorina saxatilis lives well under laboratory conditions, where it will continue to reproduce as small laboratory populations, so long as these are provided with a regular supply of algal covered rocks. Because of its brooding habit, relatively small numbers of embryos (compared to planktonic spawners such as *Mytilus*), and tolerances to salinity variation and enclosure conditions, *Littorina* is a good subject for studies of the effects of chemicals on fecundity and embryo viability (growth and development) both in the field and in the laboratory. Some preliminatry results in Figure 19.9 suggest that population differences may be considerable and can be related to environmental pollution.

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Appendix

Type of test	Test organism	Sensitivity	Initial cost	Running cost
Gonad condition index	*Mytilus*	Medium	Medium–high	Low
Genetic and related effects	*Mytilus*	Medium–high	High	Medium
Larval acute toxicity	*Mytilus*	High	Medium	Medium
Developmental abnormalities	*Littorina*	Not known	Low	Low

(including man-hours)

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