Animal standardisation for mixed species ecotoxicological studies: Establishing a laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus*

Estandarización animal para estudios ecotoxicológicos mixtos: establecimiento de un programa de reproducción en laboratorio para *Gammarus pulex* y *Asellus aquaticus*

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Palabras clave: *Asellus aquaticus*, pograma de reproducción en laboratorio, *Gammarus pulex*, tests de toxicidad.

ABSTRACT

This paper outlines how to establish a standardised laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus*. Wild *Gammarus pulex* and *Asellus aquaticus* specimens were captured from an unpolluted river source and used as founder populations for the programme. The *Gammarus pulex* and *Asellus aquaticus* founder populations were permitted to breed randomly and the subsequent offspring (F^1, F^2 and F^3 generations etc.) were available as standardised test animals for mixed species aquatic toxicity tests. The husbandry required to maintain laboratory breeding populations of *Gammarus pulex* and *Asellus aquaticus* is outlined and the animals' development cycles are discussed.

RESUMEN

Este artículo se centra en el establecimiento de un programa de reproducción para *Gammarus pulex* y *Asellus aquaticus*. Se capturaron ejemplares de estas dos especies de un río no contaminado y se usaron como poblaciones iniciales para el programa. Estas poblaciones fundadoras se dejaron reproducir libremente y la descendencia resultante (F1, F2, F3, etc.) estuvo disponible para su uso en los test de toxicidad. Se discuten los detalles para el mantenimiento de las poblaciones reproductoras de estas especies así como sus ciclos de vida.

INTRODUCTION

Toxicology has become an essential procedure for monitoring the effect of pollutants on the ecology of aquatic environments. By undertaking toxicity tests it is possible to determine what concentration(s) of a particular substance(s) has a toxic or sub-lethal effect on a range of organisms so that standards for the protection of the aquatic ecosystem can be developed (Bloor, 2009). Although, toxicity tests can be undertaken with a wide range of fish and macro-invertebrate species, a test animal's toxicological response can be influenced by a variety of parameters such as, its past life history, age, reproductive state, diet and the conditions in which the tests are being performed (Bloor, 2009). Therefore, it is imperative to obtain test animals from an unpolluted standardised environment in order to achieve a response that can be relied upon. On saying this however, research has previously been published were the animals employed in the toxicity tests have been captured from wild populations a few days/weeks before the onset of testing and the test animals life history, age etc. were unknown (for example, Green *et al*., 1988).

Test animals

The most commonly applied toxicity tests are single species assays. Although, these tests are simple and cost effective, they are also environmentally unrealistic as aquatic macro-invertebrate species and fish vary in sensitivity to both organic and chemical pollutants (Boyle, 1983). Toxicological studies should therefore, be flexible and governed by the nature of the aquatic pollutant, and its known or predicted behaviour in the environment. Those species, who's feeding habits, habitat requirements and behavioural characteristics, make them most likely to be affected by the discharge can then, be chosen (Bloor, 2009). Taking the aforementioned facts into consideration a more sophisticated approach would be to undertake mixed species assays with animals of different pollution tolerances, which would

enable pollution boundaries to be established (MacNeil *et al*., 2002) such as, a comparison of key biotic indices species.

Two such species are the amphipod and isopod crustaceans *Gammarus pulex* (water column dwellers) and *Asellus aquaticus* (inhabitants of the sediment) that are important components of the freshwater ecosystem and are commonly found together throughout the British Isles, Europe and North America. Both species are frequently used as test animals for mixed species bioassays, as they have differing responses to organic and several classes of chemical pollutants (Bloor & Banks, 2005a, 2005b, 2006a, 2006b, 2006c; Bloor *et al*., 2005, 2006; Bloor, 2009). *Gammarus pulex*, for example, is sensitive to organic pollution and a range of toxicants, such as, ammonia (Thomas *et al*., 1991) and phenol (McCahon *et al*., 1990), which are less toxic to *Asellus aquaticus* (Maltby, 1995).

Researchers have previously demonstrated that juvenile *Gammarus pulex* and *Asellus aquaticus* are the most appropriate life stage to use during acute toxicity tests as they have a greater and more reliable sensitivity (McCahon & Pascoe, 1988). Pregnant females are also highly sensitive (Bloor, 2009) but a replicated response would be difficult to achieve as all of the test animals would have to be at the same gestation stage, otherwise the animals response might be hindered or exaggerated. The *Gammarus pulex* and *Asellus aquaticus* biology has also been utilised during sub-lethal toxicity tests e.g. growth and respiration rates, reproductive behaviour/success, skewed sex ratios and locomotion (Bloor, 2009; Lloyd Mills *et al*., 2004).

Culture characteristics

Theoretically, the *Asellus* male:female birth frequency is 3:1 and 1:1 for *Gammarus* (Bloor, 2009). *Asellus aquaticus* and *Gammarus pulex* enter pre-copula prior to mating, and the male guards the female until moulting takes place and insemination becomes possible (Bertin *et al*., 2002). In pre-copula pairs, the male is larger than the female as 'large' females impede the males' locomotion and so have a higher cost energy value (Adams & Greenwood, 1983). Larger males, therefore, have many advantages over their smaller counterparts, for example, they can mate with relatively large females (who produce more eggs), (Elwood & Dick, 1990) and displace smaller males during pre-copulation (Ridley & Thompson, 1979; Jivoff & Hines, 1998).

Both macro-invertebrates have a one year life-cycle and their growth rate is accelerated by increasing temperature (Okland, 1978). Sexual maturity is reached within 130 days at 15ºC for *Gammarus pulex* (McCahon & Pascoe, 1988) and 46-60 days in *Asellus aquaticus* (Marcus *et al*., 1978). Each female will produce 1-2 broods, ensuring that a large number of

offspring are available for toxicity tests at whichever life-cycle stage is required (Bloor, 2009).

Gammarus pulex

When juveniles are released from the brood pouch they possess five segments on the primary flagellum of each antenna and this number increases as growth progresses. Although, increasing the culture temperature will enhance the growth rate (Nilsson, 1974; Welton, 1979), the number of antennal segments or body length can still be used to estimate age (to within several days), if these measurements are made at the appropriate culture temperature (McCahon & Pascoe, 1988).

Approximately, 70% of cultured juveniles survive to reach sexual maturity within 130 days at 13°C (McCahon & Pascoe, 1988), which compares favourably with the work of Hynes (1955) and Welton (1979). However, Bloor (2009) noted that no mortalities occurred amongst the cultured juveniles. Sexual maturity is reached at 14-16 antennal segments (after 10 moults), when the males genital papillae are visible and the females oostegites are fully developed (with long fringe bristles, which interlace with one another to form the brood pouch), (Bloor, 2009).

The ability of females to produce 2-5 broods containing a mean of 16 eggs (range 10-26) ensures that a large number of offspring can be reared from one group of adults. By increasing the temperature and providing excess food under laboratory conditions it is possible to culture animals throughout the year, and to reduce the time taken to reach sexual maturity (Bloor, 2009).

Asellus aquaticus

Asellus aquaticus go through five marsupial stages of development. Released juveniles pass through two post-marsupial moults before the seventh pair of thoracic appendages develops. As with *Gammarus pulex*, the number of antennal segments and body length increases with age and is dependant on temperature. However, as *Asellus aquaticus* moult their width and length increases. The maximal pereonal width of males occurs across pereonite 6, whilst that of females is across pereonite 3, with the minimum width at pereonite 1 in both sexes. Thus, size can be determined as length x average pereonal width (McCahon & Pascoe, 1988). Marcus *et al*. (1978), however, found growth to be exponential from birth to sexual maturity, which is reached in 46-60 days at 15°C when body length is 3.5 to 4.0 mm.

Each female will produce 1-2 broods and the number of eggs produced per brood increases with body length, ranging from a mean of 21 for 4 mm

females to 100 for animals of 9 mm and over although, the range within each size group is large (Steel, 1961). Brood development takes 40-60 days at 5-10ºC and a further 150-200 days is required for the production of an F^2 generation (Bloor, 2009).

Food preference

It was reported in Bloor (2009) that under laboratory conditions both *Asellus aquaticus* and *Gammarus pulex* preferred a diet of organically enriched leaves (McCahon & Pascoe, 1988), in comparison to artificially treated material (Naylor *et al*., 1989). However, when offered the choice between fungal mycelia and fungally 'conditioned' leaf material *Asellus aquaticus* preferentially feed on the mycelia, whereas, *Gammarus pulex* preferred the leaf material. The ability of *Gammarus* to maintain their energy status when feeding on poorly 'conditioned' leaves enables them to feed on freshly abscised material in the wild and therefore, exploit this limited resource before other species, such as, *Asellus aquaticus* (Graca *et al*., 1993).

The food quality of detritus has been defined in terms of chemical (e.g. nitrogen and lignin), physical (e.g. resistance) and biological (e.g. microbial biomass) parameters. High quality food has a low C:N ratio, low lignin content, low resistance and high microbial biomass (Iversen, 1974). On the basis of these parameters, 'conditioned' alder leaf material (*Alnus* spp.) would be described as high quality (Graca *et al*., 1993) and is frequently used as a food source for *Asellus aquaticus* and *Gammarus pulex* during laboratory studies, for example, Naylor & Calow (1990) and Bloor (2009).

The aim of this paper is to outline how to establish a laboratory breeding programme for *Gammarus pulex* and *Asellus aquaticus* using wild founder populations, in order to produce standardised test animals for mixed species toxicity tests.

MATERIALS AND METHODS

Animal capture and transportation to the laboratory

Specimens of *Asellus aquaticus* and *Gammarus pulex* were collected, using a hand net, from an unpolluted river source (River Itchen, Southampton, U.K.) and transported to the laboratory in river water and detritus. 1 litre cylindrical plastic containers with detachable lids (pierced with air holes) were used to transport the macro-invertebrates. Diseased and parasitized specimens were rejected, for example, those animals that appeared immo-

bile, displayed external injuries or discolouration along their body segments (*Gammarus pulex* commonly hosts the *Pomphorhynchus laevis* parasite that appears as a orange 'spot' on the animals body).

Culture and rearing aquarium system

A schematic illustration of the *Asellus aquaticus* and *Gammarus pulex* culture and rearing system is outlined in Figure 1. Two culture aquariums (one for each species) were filled with river water (from River Itchen, Southampton U.K.). The collected founder populations were added to the aquariums and allowed to adjust to the environment. Partial (10 litre) water changes were undertaken on a weekly basis (the river water was replaced with dechlorinated tap water, which was obtained via a charcoal filter unit). Once the animals had fully acclimatized to the dechlorinated tap water, a flow-through supply system was operated on a 4 hour water change over rate. The tap water was passed through a charcoal filter unit (to remove the chlorine) and stored in a header tank. Two delivery tubes were used to transfer the filtered water from the header tank to the culturing aquariums. An outlet hole was drilled into the side of each culturing aquarium (the diameter of the outlet holes and delivery tubes were equal) that acted as an overflow to ensure that the water level within each aquarium remained constant (Fig. 1).

Both visibly gravid females and pairs in pre-copula were left in the sample. On a daily basis the aquariums were observed for offspring. Once the fry was released, a 10 mm aperture pipette was used to manually collect the offspring and transfer them to the rearing aquariums (species specific aquariums). The rearing aquariums were manually filled with dechlorinated tap water (obtained from the header tank). Partial water changes (5 litre) were undertaken daily. After about 25 days, those juveniles not used in the toxicity tests were transferred to the culture aquariums. By transferring each fry to different rearing aquariums, groups of animals at different age ranges were available for toxicity tests.

The aquariums were maintained at 15^{o}C and for 16 hours per day they were illuminated with a fluorescent light (with a specification for freshwater invertebrates), to simulate on a small-scale the macro-invertebrates natural climatic conditions. The glow mimicked the thermal warmth and daytime illumination obtained from the suns radiation. Both culture and rearing aquariums were aerated. Air was pumped into each aquarium via a 20 mm^3 air stone attached to a pond pump with silicon tubing (5 mm diameter). Water filtration units were initially installed in the culture and rearing aquariums, however it was observed that both juvenile and adult specimens were prone

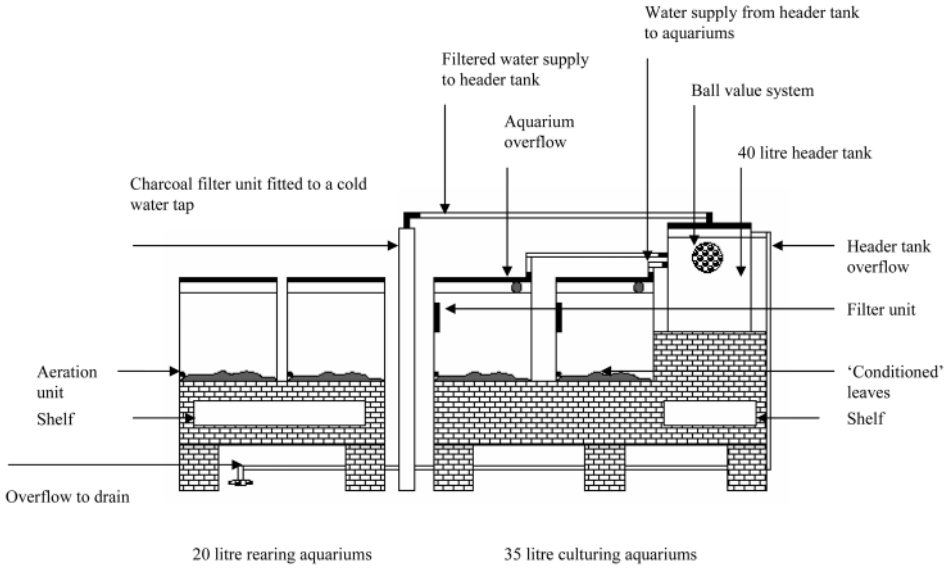

Fig. 1.—Schematic representation of the laboratory based *Asellus aquaticus* and *Gammarus pulex* culture and rearing aquarium system.
Fig. 1.—Representación esquemática del cultivo de laboratorio de *Asellus aquaticus* y *Gammarus pulex* y el sistema de acuarios.

to being sucked into the filter units causing injury and in some instances death. As such, the aquariums were manually cleaned on a daily basis. A 10 mm aperture pipette was used to extract waste detritus, taking care not to disturb the animals. An algal scrapper was used to clean the aquariums interior walls and their exterior surfaces were wiped with non-toxic cleaner.

Food

Abscised alder leaves (*Alnus* spp.) were collected during the autumn fall (from one *Alnus* spp. tree at Hillier's Arboretum, Romsey U.K.), air dried and stored. As such, the food source was standardised as all the leaves were collected from the same tree on the same day. 10 litres of river water and a handful of organic detritus were collected from an unpolluted source (River Itchen, Southampton U.K) and transfer to the laboratory in a lidded plastic container. On return to the laboratory the water and detritus was poured into a 15 litre plastic box (the box was not sealed with a lid). Handfuls of the pre-collected alder leaves were submerged in the water and mixed with the detritus ('bucket science' was used - no precise measurements). The leaves were 'conditioned' for at least 10 days. After this time and when required,

leaves were extracted from the box and placed in the aquariums (excess liquid was squeezed from the leaves to reduce the level of organic enrichment applied to the water). Additional air dried leaves were then immersed in the 'conditioning' box to replace the utilised ones.

The leaves were liberally scattered in the culture and rearing aquariums, to for fill the animals' nutritional requirements, and were replaced at regular intervals (enough leaves to cover the aquarium floor at a depth of approximately 50 mm). The juveniles were, however, supplied with 'conditioned' alder leaves for shelter and grazing but were also fed upon adult faeces that was syringed from the culture aquariums (when required), until the animals could feed entirely upon 'conditioned' leaves (after about 25 days). A small amount of floating plant material (the quantity was not measured but enough to cater for the animals' requirements without overloading the aquarium with unnecessary organic material) was also incorporated into the *Gammarus pulex* aquariums (collected from the River Itchen, Southampton U.K.).

RESULTS AND DISCUSSION

The purpose of this paper is to outline how to establish a laboratory breeding programme for *Asellus aquaticus* and *Gammarus pulex* in order to produce standardised test animals for a toxicity testing programme. Provided a sufficient number of gravid *Gammarus pulex* females (>200) were available, a large number of newly hatched individuals (500-1000) were on hand for toxicity tests. *Asellus aquaticus* are more fecund than *Gammarus pulex* and fewer gravid females (100) are required to ensure the production of sufficient juveniles (500-1500).

Within wild populations of both *Asellus aquaticus* and *Gammarus pulex* a large and fluctuating gene pool is assessable to the breeding animals, which is missing from a small-scale laboratory breeding programme. The potential difficulty with laboratory breeding programmes is that the breeding populations could be too small, which would result in a limited number of genes entering the gene pool, causing a loss of homozygosity, non-random mating, inbreeding and potentially population extinction (Bloor, 2009). As well as threatening the survival of a breeding programme, the use of inbreed animals during toxicity tests could have serious consequences on the repeatability of the assays and/or the animals' toxicological response.

It is important that a proportion of the juveniles from the F^1, F^2 and F^3 etc. generations are not used in the toxicity tests but are instead put back into the culturing tanks as procreating stock to maintain an active breeding population. This course of action would ensure that the original

population is increased and gradually replaced as they die off. Through the re-introduction of their parents' genes into the gene pool the likelihood of inbreeding and the catastrophic consequences that could result would increase. However, by simply adding a few wild animals (captured from the River Itchen, Southampton, U.K.) to the culturing tank at periodic intervals the gene pool could be increased and the likelihood of inbreeding reduced.

The animals within the breeding programme should not be subjected to stress, which could result in the animals displaying an unrepresentative response during a toxicity test (Korhonen & Lagerspetz, 1996; Lagerspetz, 2003). Stress can occur as a consequence of many things including; excessive handling, temperature, malnutrition, predation and confrontation. For example, McCahon & Pascoe (1988) designed a laboratory breeding programme in which both visibly gravid females and pairs in pre-copula were removed from the collected founder population (and subsequent generations), and transferred to a small breeding container that had small holes in the base through, which juveniles could pass after release from the brood pouch. This breeding container was then suspended in a rearing tank. In preliminary studies Bloor (2009) used this method but observation of the breeding containers showed that the animals appeared to show signs of distress, e.g. constant rapid movements, collisions with each other and aggressive behaviour. The aforementioned system was therefore, adapted to eliminate animal stress. In the revised procedure (as discussed in the methodology section of this paper), gravid females and pre-copula pairs remained in the culturing tank, and on a daily basis offspring were collect and relocated to the rearing tank (using a 10 mm diameter pipette).

Gammarus pulex and *Asellus aquaticus* have a tendency to eat their own offspring and in some cases the cadavers of other animals (McCahon & Pascoe, 1988; Bloor, 2009). During this study the offspring were separated from their parents at birth and those specimens not used during toxicity tests were returned to the culturing aquarium after 25 days. At this age the animals were able to survive nutritionally on 'conditioned' leaves (without adult faeces supplements) and were large/strong enough to defend themselves that would reduce the likelihood of the animals becoming stressed.

In laboratory breeding programmes, the density of the macro-invertebrates with in given space would be greater than those found naturally in wild stocks. As such, and given the aggressive/cannibalistic behaviour of the macro-invertebrates the animals could become stressed or injured. This potential problem was overcome by adding an excess of 'conditioned' leaves to the aquariums (enough leaves to cover the aquarium floor at a depth of approximately 50 mm), which were replaced at regular intervals to provide the animals with sufficient food and coverage to prevent aggressive behav-

iour and conflict. The water column dwellers *Gammarus pulex* were also supplied with floating plants (obtained from an unpolluted water source), which they could eat and hang off.

Although single species toxicity tests can be performed using either *Gammarus pulex* or *Asellus aquaticus*, by undertaking mixed species tests using both macro-invertebrates pollution boundaries can be established, which take into account the pollution tolerance of *Asellus aquaticus* and the sensitivity of *Gammarus pulex* (Bloor & Banks, 2005a, 2005b, 2006a, 2006b, 2006c; Bloor *et al*., 2005, 2006; Bloor, 2009). The main reasons for carrying out single species toxicity tests are the economic implications and the complexity of caring for two species (Boyle, 1983). However, *Gammarus pulex* and *Asellus aquaticus* have the same nutritional and habitat requirements, and are relatively simple and inexpensive to breed that make them ideal co-breeding species.

CONCLUSIONS

Taking into account the limitations of a toxicity testing programme without animal standardisation, the research presented here may provide valuable information for biologists, ecotoxicologists and environmental scientists. Through the establishment of a standardised breeding programme, the origin of the test animals would be known and the specimens' response comparable (if a standardised toxicity testing procedure is used). Furthermore, if mixed species toxicity tests were implemented using animals of different sensitivities e.g. *Gammarus pulex* and *Asellus aquaticus*, pollution boundaries could also be established and the integrity of a riverine community best protected.

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