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A REVIEW OF LABORATORY TECHNIQUES USED IN THE CULTIVATION OF SOIL DWELLING EARTHWORMS

Rearing earthworms under laboratory conditions is now essential due to developments in ecotoxicology and soil restoration. A review of such research for temperate, soil dwelling species is presented. Critical abiotic and biotic factors including soil moisture, temperature and pH, earthworm density and species composition are considered. Optimal values are provided for 4 species (Allolobophora chlorotica, Aporrectodea caliginosa, Aporrectodea longa and Lumbricus terrestris). Guidelines are offered for culture of these and other soil dwelling species.

Keywords: Earthworms, growth, laboratory culture, reproduction, temperate species

I. INTRODUCTION

The past 20 years have seen an expansion in applied earthworm research, for example, investigating potential in soil restoration [10], biomonitoring [25] and exotoxicology [38]. The epigeic species *Eisenia fetida* (Sav.) was and still remains the favoured ecotoxicological test species, but more recently soil dwelling species such as *Lumbricus terrestris* L. have been identified as more suitable alternatives, largely due to their intimate contact with the mineral soil. However, there is a general consensus that successful and sustainable laboratory culture of such earthworms is, if not impossible, at the least very difficult. This has led to a reliance on commercially purchased or field collected experimental subjects. The reliability of results achieved with earthworms of unknown origin or of unknown previous exposures must be questionable.

This paper offers guidance on optimal levels of abiotic and biotic factors associated with sustainable laboratory culture of temperate soil dwelling (endogeic and anecic) earthworm species (see Table 1), but does not consider rearing of epigeic species (such as *E. fetida*) as their culture requirements are simpler and have been frequently documented [16].

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II. ENVIRONMENTAL (ABIOTIC) FACTORS

Moisture Content

Most researchers report soil moisture content as a percentage of the wet soil mass [2]. This is the most practical method, but without detailed description of the soil used, it is impossible to draw valid comparisons between related works and also makes determination of optimal soil moisture values difficult. However, moisture levels of 25-30% in a loamy soil have been employed successfully by a number of researchers in the culture of a range of soil dwelling species [11].

Under laboratory conditions, loss of moisture from culture substrate through evaporation can become a problem. To combat water loss, cultures can be maintained in sealed containers with small air holes (< 1mm) for ventilation [13,24]. Several workers have determined actual water loss by weighing cultures (or equivalent control vessels) on a regular basis and replacing water as necessary [1,42]. Replacement moisture is usually applied to the soil surface.

Soil moisture can significantly influence cocoon development. Drying soils lead to cocoon dehydration which can retard embryonic development [22]. Over the last 15 years a method of incubating cocoons on or between moist filter papers in Petri dishes (or similar vessels) has been developed, with the filter papers re-hydrated as required. The filter papers can also serve as a food source for earthworm hatchlings, and for this reason hardened paper should be avoided (Whatman No 1 or 2 qualitative papers are recommended). To ensure that cocoons do not dehydrate several researchers [6] have provided excess water, so that cocoons are totally submerged. This procedure does not negatively affect cocoon development or survival of emerging hatchlings.

Temperature

All biological activities of earthworms are influenced by temperature, not only by mean values but also by extremes and fluctuations [30]. Therefore temperature control can be used to manipulate all aspects of earthworm life cycles.

At low temperatures (3-5^{o}C) hatchling development is severely impeded. For example, Butt [6] recorded weight gains for *L. terrestris* hatchlings (initial mass 0.05 g) after 16 weeks of 2.6 g at 20^{o}C compared with 0.25 g at 5^{o}C. Therefore in experiments requiring large cohorts of hatchlings of similar mass, low temperatures can be used to inhibit growth (enforced quiescence) until sufficient earthworms have hatched [37]. Cocoon development can also be manipulated by controlling temperature, as embryo development decreases with decreasing temperature, and may be inhibited at 3^{o}C with cocoons non-viable if frozen [24]. These authors recorded that *A. chlorotica* embryonic development occurred in 34-38 days at 20^{o}C compared with 400 days at 5^{o}C (with cocoon hatching inhibited at the latter). When cocoons from lower temperatures (e.g. 5^{o}C) are subsequently placed at elevated temperatures (e.g. 15-20^{o}C) they tend to hatch rapidly enabling production of a cohort of hatchlings. Several workers have maintained earthworm cocoons at 5^{o}C in order to slow development and prevent hatching [4], but minimum temperatures required for hatching are species specific, e.g. *A. caligionsa,* unlike *A. chlorotica* readily hatched at 5°C [24].

Earthworm growth and fecundity is temperature dependant and both increase with increasing temperatures to critical (lethal) thresholds. Maintenance of laboratory-reared earthworms under constant temperature conditions can overcome the influence of seasonality on behaviour and production. Under field conditions several species (e.g. *A. longa)* aestivate during the summer months and may lose reproductive condition, but under constant temperatures, earthworms have been shown to maintain both activity and reproductive

condition throughout the year. However, earthworms kept at temperatures approaching the upper tolerance limits can suffer from reproductive fatigue, and experience high death rates and loss in body mass compared with those kept under fluctuating temperatures. For example, Butt [8] maintained adult *A. chlorotica* at 10, 15 and 20°C for 12 months, with mean cocoon production rates of 9.9, 17.8 and 27.3 cocoons worm^{-1} year^{-1} respectively and equivalent adult survival of 73, 93 and 15%. A trade-off is therefore seen between production and survival. To successfully culture temperate soil dwelling species researchers have therefore tended to use a sub-optimal temperature of 15°C for rearing juveniles and incubating cocoons [19,32]. Cultures are usually maintained in temperature controlled incubators or rooms [32], but other methods of maintaining constant temperature are also available. Baker et al. [1] kept earthworm cultures in water baths while Butt et al. [12] used an insulated polythene greenhouse with sub-soil heating cables for large-scale breeding of earthworms.

Substrate

Epigeic (surface dwelling) species are usually cultured in 100% organic matter substrates, however soil dwelling species require a soil and organic matter matrix [10,15].

The use of field-collected earthworms in laboratory-based studies has led to the use of soils collected from the same area as a culture medium [14,20]. Such field-collected soils are of particular relevance if the research objectives are to study the influence of inherent soil macro- and micro-fauna on e.g. earthworm behaviour or production. However, if soil is for use only as a culture medium, then removal of non-target resident earthworms, potential predators, competitors, parasites and pathogens is preferable. As a result, researchers have employed several methods of soil preparation including: sieving and hand sorting [19], steam sterilisation [6], subjection to microwaves [28] or simply left to air dry [35].

Pre-treatment of soil, whilst desirable is time-consuming, hence an acceptable alternative, i.e. the purchase of commercial soils, has been adopted by some. Butt et al. [11] used pre-sterilised (to remove macro- and meso-invertebrates) and sieved (<6 mm) Kettering loam with an organic content of 5% and a pH of 6.4 to culture *L. terrestris*. This soil has subsequently been adopted by other researchers and proposed as a standard medium for use in toxicology tests [39].

Food

The preference for animal dung over other organic materials as a suitable feed for earthworms has been recognised since the pioneering work of Evans and Guild [17]. As a result dung of cattle [27], sheep [1] and horses [39] has been widely used in earthworm culture.

Several researchers have used fresh / semi-decomposed dung as a food source, [18]. However, fresh dung may contain potential competitors along with a resident earthworm fauna that may compromise cultures and influence experimental results. In addition, the ammonia content may adversely affect soil dwelling earthworms. To achieve a consistent and reliable food source, animal dung requires pre-treatment. Spurgeon et al. [39] froze fresh (field collected) animal dung to sterilise and maintain its nutritional value whilst Lőfs-Holmin [29] recommended keeping semi-composted cattle dung in air tight containers at 25°C for several months to kill invertebrates and earthworm cocoons.

Although animal dung is recognised as a suitable earthworm food, field collection can be a time consuming practice. Intensive large-scale production of soil dwelling earthworms has identified the need for a more consistent and abundant food source. Research led by Clive Edwards at Rothamsted in the early 1980s explored the potential of using slurry (dung and urine) as a food for earthworms [16]. Separating the solid fraction produced a consistent and abundant earthworm food source (termed separated cattle solids (SCS))

that was has been widely adopted [27,31] for use in earthworm culture. Drying SCS volatilises ammonia and also allows for short-term storage of food without microbial degradation. For use thereafter, dried SCS is rewetted, before being fed to earthworms.

Animal dung is not the only food used to culture earthworms, other organic waste materials from agriculture or industry have also proved successful. Addition of nitrogen-rich spent brewery yeast to solid paper mill residues (a potential earthworm food source itself) by Butt [7] resulted in rapid growth rates of *L. terrestris* compared to earthworms fed on paper residues alone. However, high nitrogen content does not always ensure increased production rates [4].

Particle size

Food particle size affects rates of earthworm growth and reproduction [3,4]. Boyle [5] and Lowe and Butt [33] have demonstrated that the influence of food particle size on earthworm growth is both species and life stage specific with smaller earthworms benefiting more from reduced particle size.

Position of Food

The location of food within the soil profile also has a species / life stage- specific influence on growth rates and behaviour. In general, anecic species fare best with surface application of food materials while endogeic species require food incorporated into the soil profile. However, Boyle [5] observed that both *L. terrestris* and *A. caliginosa* grew more rapidly when food was applied at the soil surface of experimental cultures compared with intimate mixing in the soil profile. It was proposed that surface applied food represented a more easily located and concentrated source even for the endogeic *A. caliginosa*.

III. BIOTIC FACTORS

The environment in which earthworms are found / kept significantly affects their growth and reproduction. In addition to this, but perhaps on a lesser scale under natural conditions, the presence of other species may be critical.

Earthworm Origin

Laboratory breeding of earthworms and manipulation of life cycles through the control of environmental conditions permits cohorts of known age and history to be produced. However, the origin and composition of "starter" cultures can significantly compromise the suitability of these earthworms and their offspring for use in experimental studies. Mass collection of certain soil dwelling species, such as *L. terrestris*, for the fishing bait market forms a major business in some countries such as Canada [41]. A worldwide trade in earthworms exists and therefore, if they are bought from a "worm breeder" it is possible that they may have originated in another country. If earthworms are field-collected, consideration should be given to their origin as differences in growth, maturation and fecundity may occur between populations of the same species from different places, as demonstrated for both *A. longa* and *Aporrectodea. rosea* (Sav.) [23,24,26]. Therefore if populations of the same species are collected from different locations, they are best cultured and used separately.

It is also important to consider that morphological variation occurs within species and this may influence breeding success. *A. caliginosa* is a highly plastic species with many morphological variants [36]. Several authors have classified the four recognised morphs as

separate species (*A. caliginosa, A. nocturna, A. trapezoides* and *A. tuberculata*) [21], however the variations that occur between the morphs may be largely phenotypic. Nevertheless, if this species is to be cultured it is advisable to separate phenotypes / species. Two morphs of *A. chlorotica* (pink and green) also exist and differences in their distribution have been recorded [34]. The morphs generally form separate populations, the green type at wet sites and the pink type in drier sites. Breeding experiments have indicated that offspring of pink and green crosses are wholly or partially male sterile [34] and therefore these morphs should also be cultured separately.

Density

Laboratory-based experiments have shown that earthworm growth, adult mass and fecundity are significantly influenced by earthworm biomass and density in culture e.g. for *A. chlorotica* [8] and for *L. terrestris* [11]. Increased density had a negative effect on growth rate and final mean earthworm mass. For *L. terrestris* the development of full reproductive capacity was also reduced at higher densities. In a 2 litre system, it was estimated that a mass in the range of 15-25 live g l^{-1} (3-5 adults) may be optimal for *L. terrestris,* while in smaller pots (0.3 litre) with a superior feed, an optimum was 20-40 g l^{-1}. This result suggests that the influence of density may be modified by other factors such as food quality.

Vessel Type

Löfs-Holmin [29] recommended that for culturing earthworms "small vessels should be preferred to large ones for ease of handling and sampling". It is also important that vessels are re-usable, easily stacked to maximise available space, have sealable lids to prevent excess loss of soil moisture and, if cultures are maintained in the light, vessels with opaque sides should be used. These recommendations have been widely adopted [24,32].

Petri dishes have been widely adopted for incubating cocoons (see Moisture section) but alternative vessels also have merits. For example, Svendsen et al. [40] incubated *L. terrestris* cocoons in 12-well tissue culture plates with moist filter paper and one cocoon in each well to allow for individual cocoons to be labelled and monitored during incubation.

Mixed Species Culture

Laboratory-based research has demonstrated that some soil dwelling earthworm species are capable of co-existence in experimental cultures [9] but also that species composition of laboratory-based cultures can affect earthworm production [20,31]. Negative interactions are species specific and thought to result from competition for resources (food and space). The intensity of interaction may largely be determined by the degree of niche overlap and is therefore most intense between species from the same ecological grouping [31]. Further research by Lowe and Butt [33] has also determined that the stage of individual earthworm development can influence both inter- and intra-specific interactions. Early growth of *L. terrestris* hatchlings was significantly greater in the presence of conspecific adults (where a high level of niche overlap would be expected) but such an advantage, possibly mediated by the availability of fragmented organic matter in the adult middens, decreased with age. In the early growth stages anecic worms may therefore be in direct competition for space and food with adult endogeic worms, something co-culture must consider.

Table 1- ***Tabela 1***

Guidelines for sustained culture of 4 species of temperate, soil dwelling earthworms
N.B. (Objectives of research might mean that some elements would not fit; e.g. if examining burrow features of *L. terrestris*)
Sugerowane metody ustawicznej hodowli 4 gatunków dżdżownic średnio i głęboko kopiących
Uwaga (Założenia badań mogą powodować, że niektóre elementy mogą nie być odpowiednie, na przykład podczas badań właściwości korytarzy L. terrestris)

Culture Parameters *Parametry hodowli*	Anecic		Endogeic	
	A. longa	*L. terrestris*	*A. chlorotica*	*A. caliginosa*
Soil Type *Typ gleby*	Loam (pre-treated to remove macro- and meso-invertebrates) *Podłoże gliniaste (przygotowane przez usunięcie makro i mezofauny)*			
Soil Depth (cm) *Głębokość gleby (cm)*	> 10	> 10	> 3	> 3
pH	6-7	6-7	6-7	6-7
Soil Moisture (%) *Wilgotność* (%)	25	25	25	25
Food *Pokarm*	Dried and rewetted animal dung (cattle or horse) *Wysuszony i powtórnie uwodniony obornik (bydlęcy i koński)*			
Food Amount / *Dawka pokarmu* [adult $^{-1}$ month^{-1} / *os. dorosły mies.$^{-1}$*]	>20 g	>20 g	>10 g	>10 g
Food location *Podanie pokarmu*	Surface Applied *Powierzchniowo*		Mixed into the soil *Wmieszanie w glebę*	
Food Particle size (mm) *Rozdrobnienie* (mm)	< 10	< 10	< 1	< 1
Temperature / *Temperatura (°C)*	15	15	15	15
Light / *Światło*	24 hr dark *ciągła ciemność*	24 hr dark *ciągła ciemność*	24 hr dark *ciągła ciemność*	24 hr dark *ciągła ciemność*
Vessel Type *Rodzaj pojemnika hodowlanego*	Sealed, opaque, preferably plastic with ventilation holes in the lid *Zamknięty, nieprzejrzysty, najlepiej plastykowy, z otworami wentylacyjnymi w pokrywie*			
Stocking Density / *Zagęszczenie* [adults dm^{-3} / *osobniki dorosłe dm^{-3}*]	4	3	10	6

IV. CONCLUSIONS

Successful laboratory-rearing of earthworms is time consuming and relies heavily on frequent monitoring and maintenance of cultures to ensure that environmental factors are kept within acceptable ranges. If a single factor is allowed to fluctuate outside of a given range, for example food is not replenished at frequent intervals or temperature control is interrupted, then earthworm production and survival will be affected.

The use of laboratory cultured earthworms instead of field collected or commercially purchased earthworms must be viewed as a significant step towards achieving reliable and replicable experimental data in ecotoxicology and also in other well established fields of

earthworm research. The production of sustainable cultures also provides researchers with the means to pursue other research areas. For example, methods for the culture of commonly found earthworm species have been developed and, with caution, these methods can be extended to other species. However, there are still a large number of temperate soil dwelling species for which no culture data exist. Culture of such species would contribute significantly to the body of basic earthworm biological and ecological knowledge.

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PRZEGLĄD TECHNIK LABORATORYJNYCH UŻYWANYCH W HODOWLI DŻDŻOWNIC

Streszczenie

Hodowla dżdżownic w warunkach laboratoryjnych jest obecnie konieczna ze względu na rozwój ekotoksykologii i biologicznych sposobów rekultywacji gleby. W pracy zaprezentowano przegląd badań nad gatunkami dżdżownic średnio i głęboko kopiących. Wzięto pod uwagę istotne czynniki abiotyczne i biotyczne takie jak wilgotność, temperatura, pH gleby, zagęszczenie i skład gatunkowy populacji dżdżownic. Zaprezentowano optymalne warunki hodowli dla czterech gatunków (Allolobophora chlorotica, Aporrectodea caliginosa, Aporrectodea longa i Lumbricus terrestris). Zaproponowano wskazówki hodowlane dla tych, jak i innych gatunków średnio i głęboko kopiących dżdżownic.

Słowa kluczowe: dżdżownice, wzrost, hodowla w laboratorium, rozmnażanie, średnio i głęboko kopiące gatunki dżdżownic