Journal of Applied Ichthyology

J. Appl. Ichthyol. 31 (2015), 1–9

ISSN 0175–8659

Received: April 5, 2014
Accepted: June 3, 2014
doi: 10.1111/jai.12645

Improving nematode culture techniques and their effects on amino acid profile with considerations on production costs

By B. H. Buck[1,2], J. Brüggemann[3], M. Hundt[4], A. A. Bischoff[5], B. Grote[1], S. Strieben[1] and W. Hagen[6]

[1]*Alfred Wegener Institute, Helmholtz Center for Polar and Marine Research (AWI), Bremerhaven, Germany;* [2]*University of Applied Science, Bremerhaven, Germany;* [3]*Institute for Marine Resources (IMARE), Bremerhaven, Germany;* [4]*Institute for Environmental Sciences, University of Koblenz-Landau, Landau, Germany;* [5]*Lehrstuhl für Aquakultur und Sea-Ranching, Agrar- und Umweltwissenschaftliche Fakultät der Universität Rostock, Rostock, Germany;* [6]*Marine Zoology, University of Bremen, Bremen, Germany*

Summary

The purpose of this study was to investigate the effectiveness of 11 different culture media for production of the free-living nematode *Turbatrix aceti*. Several other harvesting methods were tested in addition to mass production. A further focus was the investigation of amino acid alterations caused by the application of various media during the culture of *T. aceti* and two additional nematode species, *Panagrellus redivivus* and *Caenorhabditis elegans*. Finally, a cost analysis for the production of *T. aceti* was generated and its outcome compared to the production of conventional live feed organisms. Altogether 11 liquid culture media were tested for mass production of the nematode *Turbatrix aceti* using a minimum of effort in terms of labour and costs. Six harvesting methods, including filtration as well as active swimming of *T. aceti* were evaluated. Additional to the culture of *T. aceti* in four of the above-mentioned media, the nematodes *P. redivivus* and *C. elegans* were cultured on two different solid media. Cost analysis for the production of *T. aceti* includes those of the media, the equipment, as well as the labour costs for culture and harvest. An average density of approx. $30 \times 10^6 \pm 8.13 \times 10^6$ nematodes L^{-1} was achieved for *T. aceti*. The most efficient method (20 μm filtration) allowed harvesting 85.3 ± 2.7% of the nematodes from the medium without disturbing the particles. Lowest efficiency was achieved by combining sedimentation and filtration, accomplishing a harvest of 42.1 ± 5.8%. The amino acid profile of all three nematode species turned out to be both stable and very similar. Amino acid enrichment had little effect. The costs for producing one million *T. aceti* individuals ranged between 5.39 and 6.19 €, where labour costs accounted for 73 to 84% of the total production costs. In conclusion, *T. aceti* appears to be very robust, easy to handle, as well as cheaper to cultivate compared to other live-feed organisms. Therefore, its use in commercial aquaculture should be given future consideration.

Introduction

The production of fish, invertebrates and plants in aquaculture has rapidly increased in recent years due to the increasing diversification of aquaculture and the lack of knowledge about new species concerning their nutrient requirements. Aquaculture thus still depends on live food for the initial feeding period. Although substantial efforts have been invested in the development of small particle-sized formulated feeds for the culture of fish and crustacean larvae, numerous species still depend on live food to meet their nutritional requirements during the onset of exogenous feeding or even to a large extent their larval-rearing period (Zambonino-Infante and Cahu, 2001; Holt et al., 2011) The use of live food in aquaculture is currently limited to a relatively small number of species, of which nauplii of the brine shrimp *Artemia salina* and rotifers such as *Brachionus* sp. are the most common (Lavens and Sorgeloos, 1996). Rotifer cultivation is somewhat risky due to a possible collapse of the cultures (Støttrup and McEvoy, 2003) as well as the difficulty in keeping them free of contamination (Reguera, 1984), potentially leading to increased production costs. Although *Artemia* nauplii are, in principle, convenient in hatchery operations since the eggs can be stored over long time periods and are readily available when needed, their limited supply can lead to relatively high prices (Lavens and Sorgeloos, 2000). Due to the rapid growth demands of the aquaculture industry, which are barely satisfied by brine shrimp cyst producers, the search for non-*Artemia* alternatives must be an integral part of future aquaculture research (Sorgeloos et al., 2001).

In recent years several species of nematodes have been identified as possibly suitable alternatives to *Artemia* nauplii (Brüggemann, 2012). The non-parasitic soil nematode *Panagrellus redivivus* has received particular attention. This nematode can reproduce rapidly and provide large biomass yields (Ricci et al., 2003). According to Wilkenfeld et al. (1984), the production is about 30% cheaper compared to *Artemia* culture. Additionally, the fatty acid profiles and the lipid contents are extremely variable, depending on the culture media used (Schlechtriem et al., 2004a,b), in *P. redivivus* between 2.7 and 39.8% with regard to the total lipid content (Rouse et al., 1992; Schlechtriem et al., 2005). The amino acid profile of this nematode species is similar to *Artemia* (Biedenbach et al., 1989; Lavens and Sorgeloos, 1996;

Santiago et al., 2003) and to frozen zooplankton (Schlechtriem et al., 2004b).

These advantages prompted aquaculturists to evaluate the suitability of *P. redivivus* as a live food for feeding to different types of fish and/or shrimp. It has been shown that the use of *P. redivivus* in co-feeding regimes of the common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*) can enhance larval growth in comparison to larvae fed a dry diet. Kahan et al. (1980) and Schlechtriem et al. (2004a) found that *P. redivivus* led to decent survival rates in larvae of common carp (*C. carpio*). However, it must be noted that although *P. redivivus* seems to reach acceptable performance rates in feeding regimes of some fish species, most studies show that the survival and growth is lower than in fish fed with *Artemia* nauplii (see Brüggemann 2012, for details).

Regarding the feeding of shrimp larvae in contrast to fish larvae, nematode performance is shown to be equal or even superior compared to *Artemia* nauplii. Biedenbach et al. (1989) raised Pacific white shrimp (*Litopenaeus vannamei*) larvae on nematodes and showed that the larvae fed with different nematode densities grew faster or were at least similar in comparison to an *Artemia* diet. Also in Pacific white shrimp larvae, Focken et al. (2006) showed that there was little difference in survival when fed either nematodes or *Artemia* and that larvae grew almost as fast as with *Artemia* in high enough nematode densities (100–150 Ind. ml^{-1}). Wilkenfeld et al. (1984) found nematodes to be an excellent food for larvae of northern brown shrimp (*Farfantepenaeus aztecus*), northern white shrimp (*Litopenaeus setiferus*), as well as Pacific white shrimp. Culture performance of shrimp larvae could also be improved by improving the fatty acid profiles of nematodes by enrichment with different types of oils (Kumlu et al., 1998). Nematodes (+algae) led to higher survival rates in Indian prawn (*Fenneropenaeus indicus*) than with a diet of algae and *Artemia*.

Although there are a variety of free-swimming nematodes, aquaculture research has so far focused mainly on *P. redivivus*. The free-living nematode species *Turbatrix aceti* in particular seems to be suitable as a live food in aquaculture, since it is very similar in character to *P. redivivus*. These nematodes are approximately the same size (1–2 mm long, ~50 μm diameter) and are very tolerant against numerous environmental factors (Bruun, 1949). An interesting shared characteristic is the fact that they are able to produce n3-polyunsaturated fatty acids *de novo* (Rothstein and Götz, 1968; Krusberg, 1972; Schlechtriem et al., 2004b). It has been demonstrated that *T. aceti* can be digested by fish very similar to *P. redivivus*, and that this species is principally suitable as live feed in aquaculture (Hofsten et al., 1983). Furthermore, *T. aceti* is capable of swimming freely in the water column, which is a substantial advantage when feeding pelagic larvae as it attracts the fish through its wincing motion and further resembles a drifting motion that provokes fish to attack the prey. Other nematodes such as *P. redivivus* must be kept in suspension by means of aeration or directed water currents, which complicate the technical rearing requirements. Furthermore, the lack of an independent swimming ability may lead to high rates of feed loss due to the deposition of nematodes on the tank bottom. The apparent suitability of *T. aceti* as live feed, as demonstrated for decades in ornamental fish culture, and the apparent advantages in terms of its swimming ability, led us to conduct a series of experiments in order to improve the culture conditions of this species and evaluate the nutritional quality. Two different experiments regarding *T. aceti* cultivation were carried out, fostering several research topics:

1 Cultivating *T. aceti* in different media in order to identify suitable liquid media for mass production, with a focus on reproduction rates and costs.
2 Testing six types of harvesting methods to evaluate the effectiveness of harvesting nematodes using these different methods, with the explicit goal to develop and identify a simple and effective method.
3 Comparing amino acid profiles of three different nematode species (*T. aceti*, *P. redivivus*, *Caenorhabditis elegans*) to *Artemia* nauplii and rotifers, with the results giving an overview of potential similarities and differences in the amino acid composition of the aforementioned species. The results can be used to estimate the potential of nematodes to provide the required amino acids to larvae as conventional live food organisms.

Materials and methods

Nematode cultivation for identifying suitable liquid media

Turbatrix aceti was grown in horizontal Corning® cell culture bottles (volume 200 ml) each filled with 80 ml medium. The bottles were sealed with caps and with holes for a constant air supply. Inoculation was approx. 5000 nematodes per bottle. The cultures were kept at room temperature (20°C), with five replicas for each medium. Once a week nematode density was evaluated by counting a subsample (between 0.1 and 1 ml, depending on the nematode density) using a microscope. Evaporated water was replaced on a weekly basis. Tests were scheduled for 70 days or were aborted earlier when cultures collapsed. Altogether eleven media (media ID A – K) were used, the media compositions selected either from the literature or adapted according to suggestions from the literature as well as our own observations. Several different ingredients were used for the formulation of the media, e.g. cider vinegar, white vinegar, water, peptone, yeast extracts, sugar, sodium chloride, and acetic acid (see Table 1 for the exact media formulations and costs).

Harvest methods

A large (4-L) glass bottle was filled with medium E and equipped with aeration, inoculated with 6 000 000 nematodes and kept at 20°C for 1 month. Medium samples containing 2 000 000 nematodes each were removed from the culture to evaluate the harvest efficiency. This was repeated three times for each method. Six methods of harvesting nematodes were tested, such as filtration, sedimentation and decantation as well as active movement of the nematodes (see Table 2 for further details of the harvesting strategies).

Table 1
Ingredient composition of tested media for culture of *Turbatrix aceti*: individual amounts of each ingredient. In brackets: prime costs according to the fraction contained in the medium

Medium ID	Cider vinegar[1]	White vinegar[2]	Water[3]	Peptone[4]	Yeats extract[5]	Sugar	Salt (NaCl)	Acetic acid[6]	Medium costs
	[mL*L^{-1}] (€)	[mL*L^{-1}] (€)	[mL*L^{-1}]	[g*L^{-1}]	[g*L^{-1}]	[g*L^{-1}]	[g*L^{-1}]	[mL*L^{-1}]	[€*L^{-1}]
A	1000 (0.99)	0 (0.00)	0 (0.00)	6.25 (0.74)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1.73
B				12.50 (1.48)					2.47
C				25.00 (2.97)					3.96
D				0 (0.00)	25 (0.40)				1.39
E	500 (0.50)		500 (0.63)						1.53
F		500 (0.71)	0 (0.00)	25.00 (2.97)	0 (0.00)				4.18
G	250 (0.25)	750 (1.10							4.32
H	0 (0.00)	1000 (1.42)							4.39
I	100 (0.10)	0 (0.00)	900 (1.13)	0.00 (0.00)	25 (0.40)	25 (0.02)	2.75 (0.00)		1.65
J						2 (0.00)	0.3 (0.00)	30 (0.11)	1.74
K	1000 (0.99)		0 (0.00)		0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0.99
Costs	0.99 €*L^{-1}	1.42 €*L^{-1}	1.25 €*L^{-1}	0.12 €*g^{-1}	0.02 €*g^{-1}	6.5*10^{-4} €*g^{-1}	3.6*10^{-4} €*g^{-1}	3.50 €*L^{-1}	

[1]Hensel cider vinegar, W. Schoenenberger GmbH & Co. KG, Magstadt, Germany.
[2]White vinegar ('Tafelessig'), Karl Kühne GmbH & Co KG, Hamburg, Germany.
[3]Aqua dest.
[4]Peptone from soybean (enzymatic digest), AppliChem GmbH, Darmstadt, Germany.
[5]Yeast extract ('Leiber Bouillon'), Leiber GmbH, Bramsche, Germany.
[6]Acetic acid (ASC reagent > 99.7%), Sigma-Aldrich & Co. Ort, Germany.

Cost analysis

A cost analysis allowed a direct comparison of total costs for each medium. Costs were adjusted to the production of one million *T. aceti* individuals, including the harvest strategy. Also considered were costs for individual media, required equipment for the production and for the harvest of nematodes. Finally, operational costs during the daily routine and harvest were incorporated.

Similar calculations were done for the production of one million *Artemia* nauplii as well as one million *Brachionus* sp. Costs for required cysts, considering a hatching rate of 85% (according to the information given by the distributor) were used, adding the prices for water and salt. For *Brachionus* calculations the costs for the culture of the phytoplankton (used as feed for the *Brachionus* during the culture period) were included in the cost analysis. Costs for electrical power during the hatching process as well as for heating and water aeration were not included.

Measurement of amino acids for diet quality determination

Panagrellus redivivus and *C. elegans* were cultivated on oatmeal in 800 ml beaker glasses covered with perforated aluminium foil. Each species was cultured on two different media to investigate the influence of a nutrient transfer depending on the composition (100 g medium per culture, three replicas each): (i) pure oatmeal, and (ii) oatmeal enriched with 5 g peptone. Each culture was inoculated with 400 000 nematodes, which were counted according to the description above, and then left at 20°C for 1 week until harvest.

Turbatrix aceti was cultivated on four different media: C, D, J and K (see Table 1). For each medium there were three replicates with 200 ml medium in 500 ml Erlenmeyer flasks. Inoculation was again 400 000 nematodes and a 1-week culture period at 20°C. Nematodes were harvested by using method 5 (double-bottle, see Table 2) and crushed using ultrasonic sound (Branson Sonifier homogenizer system, Branson Ultrasonics Corporation). Afterwards, the nematodes were frozen and freeze-dried (Christ freeze-drying system, Martin Christ Gefriertrocknungsanlagen GmbH).

Samples were analysed according to Fitznar et al. (1999) using high-performance liquid chromatography (HPLC system: Agilent 1200 series HPLC-System, column: Phenomenex Kinetex; 125 × 4.6 mm; 2.6 μm).

Statistical analysis

All statistical tests were conducted using the statistical software package R Version 2.13.0 (R Development Core Team, 2013). Analysis of variance (ANOVA) was applied for comparisons of nematode yields at 56 days post-inoculation as well as for the statistical evaluation of different harvesting methods. Data were checked for normality of residuals (Shapiro Wilk's test) and equality of variance (Levene's test). In all cases post-hoc comparisons between treatments according to the Tukey–Kramer method were applied where appropriate.

Table 2
Description of tested harvesting methods

Method	Strategy
1	Filtration 20 μm: Media were filtered using a 20 μm mesh size sieve.
2	Sedimentation + Filtration 20 μm: Particles in the media (usually biofilms created by acetic acid bacteria) are heavier than vinegar and thus settle on the flask bottom. In contrast, *Turbatrix aceti* tend to swim near the surface, therefore separating themselves from particles. To take advantage of this behavior the media were put into 1000 ml vol. flask for 15 min. The particle-free medium was then decanted carefully and sieved with a 20 μm sieve.
3	Sedimentation + Filtration 67 μm: Procedure as in Method 2, but using a 67 μm sieve.
4	Double filtration: Medium first poured through a 100 μm sieve (to remove particles) then through a 20 μm sieve.
5	Double bottle: Screw caps of two bottles were glued together top-to-top, with a hole (1 cm ø) drilled through the caps. The caps were screwed onto a 750 ml glass bottle on one side and onto a 500 ml plastic bottle (with the bottom removed) on the other side, thus connecting the two bottles. In an upright position with the open bottom of the plastic bottle at the top, the glass bottle was filled with the medium until it reached the caps. The hole connecting the caps was closed with a plastic plug. The plastic bottle was then filled to the top with tap water and the plug carefully removed. Nematodes began to move through the hole to reach the open surface at the top of the plastic bottle. After 30 min the hole was closed again and the water decanted and sieved with a 20 μm sieve.
6	Volumetric flasks: 500 ml flasks were half-filled with medium. A 2 cm long piece of grease trap filter was placed directly above the surface. The rest of the flask was then very carefully filled with water so that no medium would pass through the filter. Nematodes began to move through the filter towards the surface. After 30 min the water was carefully decanted and poured through a 20 μm sieve.

Results

Identification of suitable liquid media for mass-culture of *Turbatrix aceti*

A total of 33 cultures (media A–K, n = 3) were cultivated; only three of these, all representing medium 'I', collapsed during the 70-day incubation period (Fig. 1). In all cultures the growth rates were highest before day 28. Some cultures grew up to 37% a day, which led to a rapid increase in nematode density. After day 28, growth rates declined slowly, even reaching negative values. Particularly after day 56, the cultures started to stagnate when maximum densities were reached, even declining in some cultures. Therefore, day 56 was used as a reference for nematode population growth performance, although in some cultures substantial growth occurred in the weeks thereafter. Densities varied depending on the media used. Pure cider vinegar (medium K) provided the lowest number of nematodes, with a maximum density of 4.0×10^6 ($\pm 0.7 \times 10^6$) individuals per litre at day 56. Highest measured values ranged at ca. 30 million nematodes L^{-1} at 70 days post-inoculation in medium 'D'. At day 56 post-inoculation, the nematode medium 'D' yield was significantly higher than any other medium except for medium 'C'. Both of these media were prepared using only cider vinegar. Although not significant, a proportional decrease in nematode yield relative to the amount of added peptone can be observed in media 'A'–'C'. The substitution of cider vinegar with 50% water led to only a slight decrease of approx. 4 million specimens $\times$ L^{-1} in nematode yield, as shown in medium 'E'.

A cost analysis was performed for all applied nematode culture media (Table 3). The costs of the media represent the minor part of total costs, ranging between 0.10 and 0.90 € per 1 million individuals. Costs for equipment and labour costs for culture and harvest represent the majority of the costs and were 0.79 €, 3.00 € and 1.50 €, respectively. Overall total costs for producing 1 million nematodes were 5.39 €

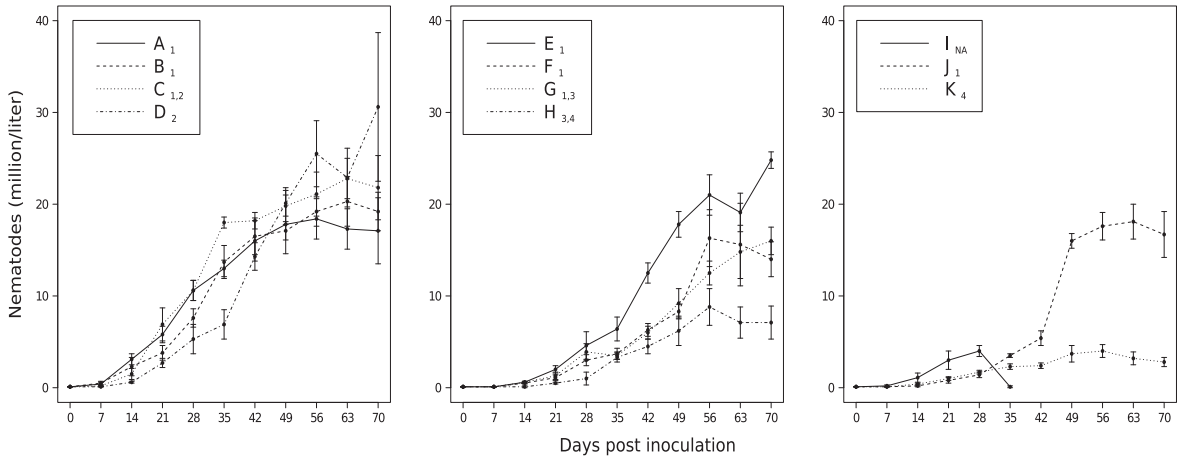

Fig. 1. Results of 70-day growth experiment of *T. aceti* with 11 different media (letters from A–K), presented as mean (±SD) (n = 3). Groups with different subscripts = significant differences in nematode yield 56 days post-inoculation

Table 3
Production costs

Medium	Costs[1]				
	Media[2]	Equipment[3]	Culture[4]	Harvest[5]	Totale
	[€]	[€]	[€]	[€]	[€]
A	0.17	0.79	3.00	1.50	5.46
B	0.23				5.52
C	0.34				5.63
D	0.10				5.39
E	0.13				5.42
F	0.46				5.75
G	0.62				5.91
H	0.90				6.19
I	---	---	---	---	---
J	0.18	0.79	3.00	1.50	5.47
K	0.45				5.74

[1]All costs based on production of one million individual nematodes.

[2]Costs of media = all ingredient costs described in Table 1.
[3]Costs for equipment contains all production costs for 5-L bottles as well as harvesting equipment required for the harvesting method 'E', for six cultures per year.
[4]Operational cost #1 = all costs originating during daily culture routine, based on time required and a 30 € hourly salary.
[5]Operational cost #2 = all original costs during culture harvest, based on time required and a 30 € hourly salary.

for the cheapest (medium D) and 6.19 € for the most expensive method (medium H).

For the *Artemia* culture, the cysts, water and salt also represent the minor part of the total costs, which were 0.82 € per 1 million hatched individuals. Operational costs represent the major part of costs and were 5.00 € and 7.50 €, respectively. Overall total costs for producing one million *Artemia* nauplii were 13.32 €, excluding the costs for equipment and electrical power during the culture.

For the *Brachionus* culture, costs for stocking individuals, water and salt also represented the minor part of the total costs, which were 5.69 €, 0.05 € and 0.02 € per 1 million individuals, respectively. The culture and harvest represent the major part of the costs of 45.00 € and 7.50 €, respectively. Overall total costs for producing one million *Brachionus* equaled a sum of 58.26 €, excluding the costs for equipment and electrical power during culture.

Harvest methods

The six tested methods led to results significantly different in harvested numbers of *T. aceti* (ANOVA; $P \leq 0.001$). All P levels presented result from Tukey's HSD test.

Method 1 (20 μm filtration) resulted in the highest number of harvested nematodes (Fig. 2). A nematode average of 85.3 ± 2.7% could be removed from the medium. Method 1 showed no significant differences compared to methods 2 ($P = 0.853$) and 4 ($P = 0.053$), but significant differences compared to methods 3 ($P < 0.001$), 5 ($P = 0.002$) and 6 ($P = 0.017$). Method 2 (sedimentation + 20 μm filtration) also resulted in high numbers of harvested nematodes. On average, 78.9 ± 3.9% of nematodes could be removed from the medium. Method 2 showed significant differences

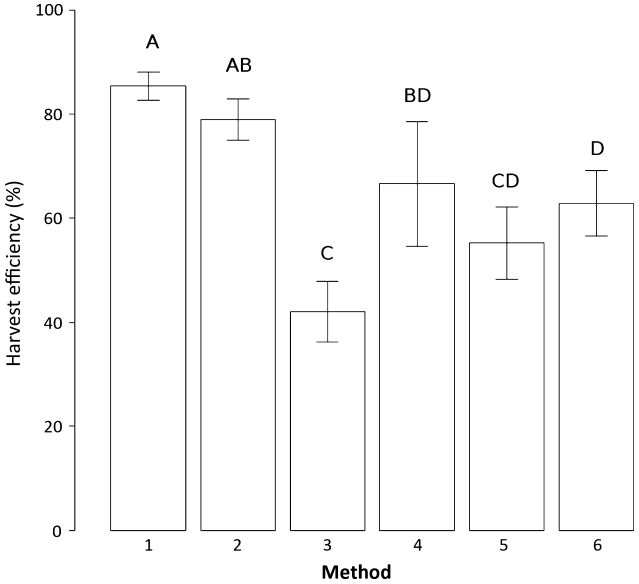

Fig. 2. Efficiency of harvesting methods 1–6; subscripts a–d = significant differences in harvesting efficiency [Tukey–Kramer *post hoc* test ($P < 0.05$) ($n = 3$)]

compared to methods 3 ($P < 0.001$) and 5 ($P = 0.015$), but no differences compared to methods 4 ($P = 0.310$) and 6 ($P = 0.116$). Method 3 (sedimentation + 67 μm filtration) reached, on average, the lowest efficiency of 42.1 ± 5.8% and showed significant differences compared to methods 4 ($P = 0.010$) and 6 ($P = 0.029$) but no significant difference compared to method 5 ($P = 0.253$). Method 4 (double filtration) reached a medium efficiency of 66.6 ± 12.0% and showed no significant differences compared to methods 5 ($P = 0.392$) and 6 ($P = 0.983$). Method 5 (double bottle) had a low efficiency of 55.2 ± 7.0% and showed no significant difference compared to method 6 (volumetric flask) ($P = 0.755$), which resulted in a medium efficiency of 62.9 ± 6.3%. Nematodes remaining in the medium were almost exclusively young and smaller individuals.

Amino acid profiles of nematodes cultured in different media

Amino acid profiles of *C. elegans*, *P. redivivus* as well as *T. aceti* did not show any significant differences (ANOVA; $P \geq 0.05$) when cultured in different media. For the first two species, the addition of peptone resulted in virtually identical amino acid profiles after a 1-week culture. Similarly, different media compositions for *T. aceti* showed little effect on amino acid compositions, which appeared to be very stable (Table 4).

Although *C. elegans* and *P. redivivus* were cultured on a starch-based medium, whereas *T. aceti* was grown on a vinegar-based amino acid medium, the profiles were almost identical among these species. The amino acids with the largest represented fractions in the different nematode species were the asparagine and aspartic acid (Asx) combined. The amounts of glutamine and glutamic acid (Glx) combined were followed by alanine (Ala), leucine (Leu), valine (Val) and isoleucine (Ile). Their fractions ranged from around 6 to 19% for

Table 4
Amino acid profile of *C. elegans*, *P. redivivus* and *T. aceti* grown on different media

	Asx[1]	Glx[2]	Ser	Thr	His	Gly	Arg	Ala	Gaba	Tyr	Aba	Val	Met	Trp	Phe	Ile	Leu
C. elegans, oatmeal	19.1 ± 6.5	12.3 ± 4.4	5.4 ± 1.8	4.8 ± 1.7	nd	9.6 ± 3.4	5.5 ± 2.0	12.1 ± 4.3	nd	3.1 ± 1.2	nd	7.8 ± 2.6	2.3 ± 1.32	nd	4.06 ± 1.4	5.8 ± 2.0	8.3 ± 2.9
C. elegans, oatmeal + peptone	18.9 ± 9.9	12.2 ± 6.0	5.9 ± 1.9	4.7 ± 2.5	nd	9.7 ± 4.3	5.3 ± 2.7	11.9 ± 5.2	nd	3.1 ± 1.7	nd	7.8 ± 4.3	2.4 ± 1.4	nd	4.0 ± 2.3	5.8 ± 3.4	8.2 ± 4.6
P. redivivus, oatmeal	18.3 ± 1.4	13.2 ± 2.3	5.1 ± 0.2	4.4 ± 0.3	nd	9.8 ± 0.9	5.8 ± 0.5	12.1 ± 1.0	nd	3.1 ± 0.3	nd	7.6 ± 0.6	2.8 ± 0.3	nd	4.0 ± 0.4	5.7 ± 0.5	8.0 ± 0.7
P. redivivus, oatmeal + peptone	18.2 ± 3.6	13.6 ± 1.5	5.3 ± 0.9	4.5 ± 0.8	nd	9.5 ± 1.7	5.5 ± 0.9	13.3 ± 4.0	nd	3.0 ± 0.4	nd	7.4 ± 1.0	2.6 ± 0.7	nd	3.9 ± 0.4	5.5 ± 0.8	7.8 ± 0.8
T. aceti, medium K	18.7 ± 3.0	13.0 ± 2.2	5.0 ± 0.8	4.5 ± 0.8	nd	10.4 ± 2.1	5.7 ± 1.0	11.9 ± 1.7	nd	2.8 ± 0.5	nd	7.7 ± 1.4	2.4 ± 0.6	nd	3.8 ± 0.8	5.8 ± 1.2	8.2 ± 1.6
T. aceti, medium C	19.5 ± 0.4	11.1 ± 2.6	5.2 ± 0.2	4.7 ± 0.2	nd	9.4 ± 0.3	5.8 ± 0.2	11.4 ± 0.5	nd	3.1 ± 0.1	nd	8.3 ± 0.1	2.5 ± 0.2	nd	4.2 ± 0.1	6.4 ± 0.2	8.6 ± 0.3
T. aceti, medium D	18.5 ± 1.7	15.1 ± 1.5	4.9 ± 0.5	4.4 ± 0.4	nd	9.9 ± 1.0	5.5 ± 0.6	11.6 ± 1.9	nd	2.7 ± 0.3	nd	7.6 ± 0.7	2.5 ± 0.4	nd	3.8 ± 0.3	5.7 ± 0.5	7.8 ± 0.6
T. aceti, medium J	18.8 ± 5.4	15.8 ± 2.9	5.4 ± 1.3	4.3 ± 1.3	nd	9.6 ± 2.5	6.0 ± 1.7	10.2 ± 3.5	nd	2.6 ± 1.0	nd	7.5 ± 2.7	1.3 ± 0.9	nd	3.9 ± 1.4	5.9 ± 2.0	8.7 ± 2.7
Artemia sp. (Appelbaum, 1979)	10.5	13.6	6.4	4.8	2.3	4.8	7.1	5.5	nd	4.4	nd	5.2	2.3	nd	4.2	4.4	7.6
Artemia sp. (Watanabe and Kiron, 1994)	11.2	12.9	6.7	2.5	1.9	5.0	7.3	6.0	nd	5.4	nd	4.7	1.3	nd	4.9	3.8	8.9
Brachionus rotundiformis (Aragão et al., 2004)	13.1 ± 0.4	12.1 ± 0.2	6.5 ± 0.2	4.3 ± 0.1	0.9 ± 0.1	5.3 ± 0.0	5.0 ± 0.1	7.4 ± 0.1	nd	5.2 ± 0.8	nd	8.5 ± 0.0	3.9 ± 0.1	nd	5.4 ± 0.2	4.6 ± 0.3	7.8 ± 0.2

Medium K = pure cider vinegar; medium C = pure cider vinegar and 25 g L^{-1} peptone; medium D = pure cider vinegar and 25 g L^{-1}yeast extract; medium J = 10% cider vinegar solution with water added, as well as 25 g L^{-1} yeast extract, 2 g L^{-1} sugar, 0.3 g L^{-1} salt and 30 ml L^{-1}acetic acid; nd = not detected.
Values presented as means (±SD) in % of overall quantified content of amino acids (n = 3).
[1]During hydrolysis Asn converted to Asp, Asx are both combined.
[2]During hydrolysis Gln converted to Glu. Glx are both combined. For Met, Trp and Lys the analysing method was invasive.

the above-mentioned amino acids. On average, they accounted for around two thirds of the amount of detected amino acids. In comparison to *Artemia* nauplii and rotifers, the amino acid profiles (provided by other studies, see Table 4) appeared to be similar. However, there were some differences. In particular, glycine (Gly) and alanine (Ala) levels in nematodes were almost twice as high as in both *Artemia* and rotifers. The arginine (Arg) content was almost identical in nematodes and rotifers, while it was slightly higher in *Artemia*. Nematodes contained slightly less tyrosine (Tyr) than the *Artemia* and rotifers. Histidine (His) content was low in *Artemia* and rotifers, and not detected in nematodes.

Discussion

Mass culture of *Turbatrix aceti*

The obtained densities in cultures of *T. aceti* are sufficient for the mass-culture and hatchery use of this species in aquaculture. However, they cannot compete with the values reported for the mass-culture of *P. redivivus* or *Panagrolaimus* sp., which can yield up to 291 000 individuals per gram of medium in *P. redivivus* (Ricci et al., 2003) and 238 000 individuals per millilitre of medium in *Panagrolaimus* sp. (Honnens and Ehlers, 2013). Compared to our study, those values exceed *T. aceti* yields by around 10-fold. With pure cider vinegar the nematodes grew steadily and cultures were stable, but the amounts produced were comparatively low. The main reason for this is probably the small content of nutrients and, particularly, proteins in the medium, which does not allow a high nematode biomass. Cultures based on medium 'I' became quickly contaminated and collapsed after inoculation, which could be observed by the build-up of fungi at the medium surface. The reason for this instant contamination was most likely the lack of sufficient acetic acid, since the medium contained 10% cider vinegar (with 5% acid). The low amount of acetic acid and the corresponding rise in pH might have enabled other bacteria than the acetic acid bacteria to grow in the medium. As shown in medium 'J', which was very similar to medium 'I', the amount of acetic acid seemed to be the main determinant in controlling microbiological activity of the growth medium. When acetic acid concentrations were sufficient, the cultures were stable and no off-odours or visible microbiological contamination were observed. Regarding food preservation techniques for human consumption, it has been shown that acetic acid has a toxic effect on a variety of bacteria, exceeding the inhibitory function exerted by only a decrease in pH (Levine and Fellers, 1940). Due to its toxic activity towards bacteria, acetic acid has also been used as a natural antibiotic in the treatment of infections of the ear (Thorp et al., 1998). Therefore, the production of live feed organisms in a medium that is rich in acetic acid has several advantages in comparison to other production methods, as contamination by microorganisms can be prevented effectively without expensive laboratory equipment.

Production cost considerations

All investigated media, apart from medium 'I', were principally suitable for nematode production, but large differences were found in terms of production costs per medium for producing 10^6 nematodes (Table 3). However, a total production price per 10^6 nematodes was relatively similar, as costs for equipment and the costs for labour and harvesting were substantially higher than the media costs. Based on the cost analysis of all media, the medium 'D' at 5.39 € was most cost-efficient in terms of nematode yield per capital invested. Also medium 'J', which contained only 10% cider vinegar at a price of 5.47 € might be of interest, as costs could probably be further reduced by using industrial-type acetic acid in large quantities. Unfortunately, no detailed cost information on mass culturing methods of *P. redivivus* or *Panagrolaimus* sp. is available. Economic comparison to conventional live feed organisms is difficult, as costs are mainly influenced by the amount of labour connected to the production of these organisms. Labour costs for *T. aceti* in comparison to *Artemia* will decrease with increasing production numbers, as *Artemia* nauplii have to be produced on a daily basis and therefore require the same daily labour input. The production of *T. aceti* requires the largest input of labour at the start of a production cycle in preparing the medium and culture, but requires no further labour at later stages. Furthermore, it is possible to upscale nematode production very easily, making it possible to produce large quantities of *T. aceti*; the subsequent workload is limited mainly to harvesting. A similar situation as for *Artemia* is for the production of rotifers, which are even more labour-intensive than *Artemia* since the rotifers need a regular daily food supply, normally phytoplankton. The phytoplankton must be either collected from the wild or cultured on its own, which is also labour consuming. Assuming a doubling time for *Brachionus* of <2 days, they still need either a large culture volume or time until the required amount of *Brachionus* is produced. Both cases result in elevated production costs compared to the culture of nematodes.

Nematode harvesting

In terms of tested harvesting methods, we found the most recommendable concept to be method number 2 (sedimentation + filtration 20 μm). Aeration in the tank can be turned off to allow particles to settle. After 30 min the desired quantity of the culture can be collected near the surface. During harvest, the medium should be collected in an extra container for reuse (also the case in all other described methods). As our culture experiments show, the lag-phase of most cultures is between 56 and 70 days post-inoculation, a time when the nematodes should be harvested and the media exchanged.

Amino acid profile of nematode species

The amino acid profile of the cultured nematodes appeared to be extremely stabile. This is in accordance with other studies in which nematodes were grown on different media to evaluate effects on the amino acid composition. Schlechtriem et al. (2004c) grew nematodes (*P. redivivus*) on oatmeal or an artificial medium made of cornstarch, yeast extract, peptone, glucose, sodium chloride and water. These completely different media had virtually no effect on the amino acid

profile. In the same study, the amino acids of zooplankton were also analysed; the results showed no significant differences between the nematodes and zooplankton. Media enrichment with *Spirulina* sp. bacteria also had no significant effect on the amino acid profile (de Lara et al., 2007). Due to the close resemblance of the amino acid profiles with other live food organisms such as *Artemia* nauplii and nematodes (Appelbaum, 1979; Watanabe and Kiron, 1994), as well as rotifers (Aragão et al., 2004; see Table 4), it is reasonable to assume that nematodes can provide larvae with the required amino acids. The amino acid profiles in all three nematode species were virtually identical, and *P. redivivus* has been successfully tested on various fish and crustacean larvae, such as common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), pacific white shrimp (*Litopenaeus vannamei*), brown shrimp (*Farfantepeneaeus aztecus*) and northern white shrimp (*Litopenaeus setiferus*). This leads to the conclusion that the amino acid composition of *T. aceti* should be a suitable diet for the above-mentioned fish and shrimp larvae.

Acknowledgements

This project was funded by the Federal State of Bremen (Germany): The Senate for Environmental Affairs, Construction, Transport and Europe within the funding program 'Applied Environmental Research - AUF' (FV 198). Many thanks also go to Prof Boris Koch (AWI, University of Applied Science Bremerhaven) and to Prof Stefan Wittke (University of Applied Science) for their support.

References

Appelbaum, S., 1979: The suitability of alkan yeast (hydrocarbon grown yeast) as a first nutrient for *Coregonus albula* (L.) fry. In: Finfish nutrition and fishfeed technology. Vol. 1. J. E. Halver and K. Tiews (Eds). Heenemann GmbH & Co, Berlin, pp. 515–524.

Aragão, C.; Conceicão, L. E. C.; Dinis, M. T.; Fyhn, H. J., 2004: Amino acid pools of rotifers and *Artemia* under different conditions: nutritional implications for fish larvae. Aquac. **234**, 429–445.

Biedenbach, J. M.; Smith, L. L.; Thomsen, T. K.; Lawrence, A. L., 1989: Use of the nematode *Panagrellus redivivus* as an *Artemia* replacement in a larval penaeid diet. J. World Aquac. Soc. **20**, 61–71.

Brüggemann, J., 2012: Nematodes as live food in larviculture – a review. J. World Aquac. Soc. **43**, 739–763.

Bruun, A. F., 1949: The use of nematodes as food for larval fish. J. Conseil **16**, 96–99.

Fitznar, H. P.; Lobbes, J. M.; Kattner, G., 1999: Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with *o*-phthaldialdehyde and *N*-isobutyrylcysteine in seawater and fossil samples (mollusks). J. Chromatogr. A **832**, 123–132.

Focken, U.; Schlechtriem, C.; von Wuthenau, M.; Garcia-Ortega, A.; Puello-Cruz, A.; Becker, K., 2006: *Panagrellus redivivus* mass produced on solid media as live food for *Litopenaeus vannamei* larvae. Aquac. Res. **37**, 1429–1436.

Hofsten, A. V.; Kahan, D.; Katznelson, R.; Bar-El, T., 1983: Digestion of free-living nematodes fed to fish. J. Fish Biol. **23**, 419–428.

Holt, G. J.; Webb, K. A.; Rust, M. B., 2011: Microparticulate diets: testing and evaluating success. In: Larval fish nutrition. G. J. Holt (Ed.). Wiley-Blackwell, Oxford, pp. 353–373.

Honnens, H.; Ehlers, R. U., 2013: Liquid culture of *Panagrolaimus* sp. for use as food for marine aquaculture shrimp and fish species. Nematology **15**, 417–429.

Kahan, D.; Bar-El, D.; Brandstein, Y.; Rigbi, M.; Oland, B., 1980: Free-living nematodes as a dietary supplement in the rearing of fish fry in hatcheries. General Fisheries Council of the Mediterranean. Stud. Rev. **57**, 67–78.

Krusberg, L. R., 1972: Fatty acid composition of *Turbatrix aceti* and its culture medium. Comp. Biochem. Physiol. B **41**, 89–98.

Kumlu, M.; Fletcher, D. J.; Fisher, C. M., 1998: Larval pigmentation, survival, and growth of *Penaeus indicus* fed the nematode *Panagrellus redivivus* enriched with astaxanthin and various lipids. Aquac. Nutr. **4**, 193–200.

de Lara, R.; Castro, T.; Castro, J.; Castro, G., 2007: Cultivo del nematode *Panagrellus redivivus* (Goodey, 1945) en un medio de avena enriquecida con S*pirulina* sp. Rev. Biol. Mar. Oceanogr. **42**, 29–36.

Lavens, P.; Sorgeloos, P. (Eds), 1996: Manual on the production and use of live food for aquaculture. FAO Fisheries Technical Paper 361. 295 pp.

Lavens, P.; Sorgeloos, P., 2000: The history, present status and prospect of the availability of *Artemia* cysts for aquaculture. Aquac. **181**, 397–403.

Levine, A. S.; Fellers, C. R., 1940: Chloride and sucrose in the presence of sodium acid upon microorganisms. J. Bacteriol. **40**, 255.

R Development Core Team 2013: R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org. (accessed on 10 March 2014).

Reguera, B., 1984: The effect of ciliate contamination in mass cultures of the rotifer *Brachionus plicatilis* O.F. Müller. Aquac. **40**, 103–108.

Ricci, M.; Fifi, A.; Ragni, A.; Schlechtriem, C.; Focken, U., 2003: Development of a low-cost technology for mass production of the free-living nematode *Panagrellus redivivus* as an alternative live food for first feeding fish larvae. Appl. Microbiol. Biotechnol. **60**, 556–559.

Rothstein, M.; Götz, P., 1968: Biosynthesis of fatty acids in the free-living nematode *Turbatrix aceti*. Arch. Biochem. Biophys. **126**, 131–140.

Rouse, D. B.; Webster, C. D.; Radwin, I. A., 1992: Enhancement of the fatty acid composition of the nematode *Panagrellus redivivus* using three different media. J. World Aquac. Soc. **23**, 89–95.

Santiago, C. B.; Gonzal, A. C.; Ricci, M.; Harpaz, S., 2003: Response of bighead carp *Aristichthys nobilis* and Asian catfish *Clarias macrocephalus* larvae to free-living nematode *Panagrellus redivivus* as alternative feed. J. Appl. Ichthyol. **19**, 239–243.

Schlechtriem, C.; Ricci, M.; Focken, U.; Becker, K., 2004a: Mass produced nematodes *Panagrellus redivivus* as live food for rearing carp larvae: preliminary results. Aquac. Res. **35**, 547–551.

Schlechtriem, C.; Tocher, D. R.; Dick, J. R.; Becker, K., 2004b: Incorporation and metabolism of fatty acids by desaturation and elongation in the nematode, *Panagrellus redivivus*. Nematology **6**, 783–795.

Schlechtriem, C.; Ricci, M.; Focken, U.; Becker, K., 2004c: The suitability of the free-living nematode *Panagrellus redivivus* as live food for first-feeding fish larvae. J. Appl. Ichthyol. **20**, 161–168.

Schlechtriem, C.; Focken, U.; Becker, K., 2005: Digestion and assimilation of the free-living nematode *Panagrellus redivivus* fed to first-feeding coregonid larvae: evidence from histological and isotopic studies. J. World Aquac. Soc. **36**, 24–31.

Sorgeloos, P.; Dhert, P.; Candreva, P., 2001: Use of the brine shrimp *Artemia* spp. in marine fish larviculture. Aquac. **200**, 147–159.

Støttrup, J.; McEvoy, L. (Eds), 2003: Live feeds in marine aquaculture. Blackwell Publishing, Oxford, pp. 145–205.

Thorp, M. A.; Kruger, J.; Oliver, S.; Nilsson, E. L.; Prescott, C. A., 1998: The antibacterial activity of acetic acid and Burow's

solution as topical ontological preparations. J. Laryngol. Otol. **112**, 925–928.

Watanabe, T.; Kiron, V., 1994: Prospect in larval fish dietetics. Aquac. **124**, 223–251.

Wilkenfeld, J. S.; Lawrence, A. L.; Kuban, F. D., 1984: Survival, metamorphosis and growth of penaeid shrimp larvae reared on a variety of algal and animal foods. J. World Maricult. Soc. **15**, 31–49.

Zambonino-Infante, J. L.; Cahu, C. L., 2001: Ontogeny of the gastroinstestinal tract of marine fish larvae. Comp. Biochem. Physiol. **130**, 477–487.

Author's address: Bela H. Buck, Alfred Wegener Institute Helmholtz Center for Polar and Marine Research (AWI), Am Handelshafen 12, D-27570 Bremerhaven, Germany. E-mail: Bela.H.Buck@awi.de