Projektnr: 28110-34

Abschlussbericht

FeeDH-A-Shrimp

Fermentation der Ω-3-Fettsäure Docosahexaensäure (DHA) mit heterotrophen Algen zur Anreicherung von Nematoden für die Fütterung von Shrimplarven in der Aquakultur

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Projektkennblatt
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Antragstitel **Fermentation der 12-3-Fettsäure Docosahexaensäure (DHA) mit heterotrophen Algen zur Anreicherung von Nematoden für die Fütterung von Shrimplarven in der Aquakultur**

Stichworte FeeDH-A-Shrimp

Laufzeit	Projektbeginn	Projektende	Projektphase(n)
36 Monate	**01. Oktober 2011**	**30. September 2014**	

Zwischenberichte

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Zielsetzung und Anlass des Vorhabens

DHA (Docosahexaensäure) ist eine 52-3-Fettsäure, die für die Ernährung vieler Organismen essentiell ist. In der marinen Aquakultur spielt sie eine besondere Rolle für die optimale Versorgung von Shrimplarven in den ersten 3-4 Lebenswochen. Shrimps, z.B. der Black Tiger Shrimp (*Penaeus monodon*), ernähren sich in den ersten Lebenswochen überwiegend räuberisch und sind auf lebende Beute angewiesen. Fadenwürmer (Nematoden) können als Futterorganismen eingesetzt werden. Vorteil von Nematoden ist die Möglichkeit einer biotechnologischen Massenvermehrung in Bioreaktoren, bei dem sie mit DHA und anderen Fettsäuren angereichert werden, die sie nicht selbst bilden können. Einzelligen Algen wie *Crypthecodinium chytrium* und *Schizochytrium* sp. bilden ca. 20% DHA in der Trockenmasse und sind dafür eine hervorragende Quelle. Diese Algen sind besonders interessant, die sie kein Licht benötigen, also heterotroph leben und damit in Bioreaktoren hergestellt werden können. Ziel des Projektes ist die Entwicklung eines mit DHA angereicherten Nematodenprodukts, das sich für die Fütterung von Shrimp-Larven eignet. Als DHA-Quelle dienen die heterotrophen Algen. Die Verfahrenstechnik zur Produktion der Algen und Nematoden soll optimiert werden und die Eignung der angereicherten Nematoden in Fütterungsversuchen geprüft werden.

Ergebnisse und Diskussion

Während des Projektes entwickelte die e-nema GmbH die Algenkultur mit *Crypthecodinium cohnii* im Bioreaktor bis zum industriellen Maßstab. Für die Ernte der Algen können Zentrifugen verwendet werden und für die Lagerung wurde ein Sprühtrocknungsverfahren entwickelt, ohne dass dabei signifikante Veränderungen bei der Lipidzusammensetzung auftreten und die essentiellen Fettsäuren erhalten bleiben. Des Weiteren wurde erfolgreich ein Hochdruck-Homogenisator zum Aufschluss der Algen zur Gewinnung der Fettsäuren und Verfütterung an die Nematoden (*Panagrolaimus* sp.) getestet. Die e-nema GmbH entwickelte auch ein Flüssigkulturverfahren für Panagrolaimus sp. bis zum Industriemaßstab. Die Nematoden können nach der Produktion bei 4°C und 53% rel. Luftfeuchtigkeit gelagert. Über einen Lagerungszeitraum von 5 Monaten zeigten das Fettsäureprofil und die Überlebensrate der Nematoden keine Einbußen. Die verwendeten Trocknungs- und Lagerungsmethoden eignen sich allerdings noch nicht für den Industriemaßstab.

Die Christian-Albrechts-Universität zu Kiel (CAU) hat die Fütterung der Nematoden mit *C. cohnii* getestet und weiterentwickelt. Es konnte gezeigt werden, dass nur ein geringer Anteil intakter *C. cohnii* Zellen von den Nematoden aufgenommen werden kann. Dadurch wurde zunächst ein sehr hoher Algenanteil im Futter benötigt, um zufriedenstellende Anreicherungserfolge zu erzielen. Dies konnte durch den vorangehenden Aufschluss der Algenzellen erheblich verbessert werden. Die CAU entwickelte auch die Trocknungs- und Lagerungsmethode für die angereicherten Nematoden. Der Gehalt an DHA und anderen essentiellen Fettsäuren konnte dabei fast vollständig erhalten werden.

Schließlich wurden mehrere Versuchsreihen zur Fütterung von Shrimplarven (*Litopenaeus vannamei*) im Nationalen Polytechnischen Institutes in La Paz (Centro de Investigaciones Biológicas del Noroeste S. C., Mexiko) durchgeführt. Bei den ersten Versuchsreihen reichte die verwendete Nematodendichte nicht aus, um ähnlich gute Ergebnisse hinsichtlich des Wachstums wie bei der Fütterung mit Artemien zu erzielen. Es konnte aber gezeigt werden, dass die Shrimplarven die angereicherten Nematoden fressen. PL1-Larven, die ausschließlich mit angereicherten Nematoden gefüttert wurden, hatten einen mehr als doppelt so hohen DHA-Gehalt als die Larven, die mit Artemien gefüttert wurden. Die Nematodendichte wurde im 2. Versuch erhöht und außerdem wurde der Fütterungszeitpunkt für die Fütterung mit Nematoden um 2 Tage vorverlegt. Artemienlarven sind zu dieser Zeit noch zu groß für die Shrimplarven. In der Folge konnten hinsichtlich Wachstum, Überlebensrate und DHA-Gehalt der Shrimplarven gleich gute oder bessere Ergebnisse als bei der Fütterung mit Artemien erzielt werden. Bis zum Mysis I Stadium wurde mit der Fütterung von Nematoden signifikant besser Ergebnisse erzielt als mit Artemien, selbst mit vorher nicht angereicherten Nematoden. Die Verwendung von Nematoden bis zum Mysis I Stadium erwies sich als ökonomischer als die Verwendung von Artemien. Bei der weiteren Fütterung bis zum PL1 und PL6 Stadium war die Fütterung mit Artemien und angereicherten Nematoden gleichwertig. Allerdings müssen zunehmend höhere Nematodendichten angewendet werden. Ob eine Fütterung mit Nematoden ab dem Mysis I Stadium eine ökonomisch sinnvolle Variante darstellt, muss deshalb noch in weiteren Praxisversuchen getestet werden.

Öffentlichkeitsarbeit und Präsentation

Eine erste Poster-Präsentation der Ergebnisse wurde bei dem 5. Büsumer Fischtag (http://www.aquaculture.uni-kiel.de/SitePages/Fischtage.aspx) im mariCUBE für europäische und kanadische Fachleute gegeben. Die Veröffentlichung der Fütterungsversuche in wissenschaftlichen Zeitschriften ist z. Zt. noch in Arbeit und wird Anfang 2015 geschehen.

Fazit

Aufgrund der im Projekt erzielten Ergebnisse ist die Vermarktung von Nematoden für die Aquakultur von Shrimps sehr erfolgversprechend. Aus diesem Grund entwickelt die e-nema GmbH nach Abschluss des DBU Projektes die Flüssigkultur der Nematoden und ihre Anreicherung und Trocknung im Industriemaßstab weiter. Weitere Praxisversuche in Zusammenarbeit mit dem Nationalen Polytechnischen Institutes in La Paz werden im Frühjahr 2015 durchgeführt.

Inhalt

ZUSAMMENFASSUNG

Ziel des Projektes ist die Entwicklung eines mit DHA angereicherten Nematoden-produkts, das sich für die Fütterung der Larven von penaeiden Shrimps eignet. Als DHA-Quelle für diese Anreicherung sollten dabei in Bioreaktoren vermehrte heterotrophe Algen verwendet werden. Die Verfahrenstechnik zur Produktion der Algen und Nematoden sollte optimiert und die Eignung der angereicherten Nematoden in Fütterungsversuchen geprüft werden. Idealer Weise sollte die Fütterung angereichter Nematoden die Verwendung von Artemia-Larven bei der Aquakultur von Shrimps ersetzen. Die Verwendung von Artemia-Larven ist teuer und Arbeits-aufwendig. Zudem können Artemien, da sie ebenfalls zu den Krustazeen gehören, u. a. Viruserkrankungen auf Shrimps übertragen.

Während des Projektes entwickelte die e-nema GmbH die Algenkultur mit *Crypthecodinium cohnii* im Bioreaktor bis zum industriellen Maßstab. Im 500-l-Bioreaktor konnte eine Algentrockenmasse von 38 g/l und eine DHA-Menge von 4,1 g/l erzielt werden. Für die Ernte der Algen können Zentrifugen verwendet werden und für die Lagerung wurde ein Sprühtrocknungsverfahren entwickelt, ohne dass dabei signifikante Veränderungen bei der Lipidzusammensetzung auftreten und die essentiellen Fettsäuren erhalten bleiben. Versuche des Projektpartners (Inst. f. Phytopathologie, Christian-Albrechts-Universität zu Kiel) hatten gezeigt, dass die Anreichung der Nematoden mit DHA besser funktioniert wenn die Algenzellen vorher aufgeschlossen werden. Hierfür testete die e-nema GmbH einen sogenannten Hochdruck-Homogenisator, ein Verfahren, welches sich auch im industriellen Maßstab verwenden ließe.

Des Weiteren entwickelte die e-nema GmbH das Flüssigkulturverfahren für den Nematoden (*Panagrolaimus* sp.) bis zum Industriemaßstab. In kleinen Bioreaktoren bis 30 l konnten Nematodendichten bis zu 875.000 ml^{-1} erzielt werden. In einem ersten Versuch im 500-l-Bioreaktor wurden allerdings nur 89.000 ml^{-1} erzielt. Die Nematoden wurden dann bei 4°C und 53% rel. Luftfeuchtigkeit gelagert. Über einen Lagerungszeitraum von 5 Monaten zeigten das Fettsäureprofil und die Überlebensrate der Nematoden keine Einbußen. Für die Trocknung und Lagerung der angereicherten Nematoden muss allerdings erst noch ein für den Industriemaßstab geeignetes Verfahren entwickelt werden.

Die Christian-Albrechts-Universität zu Kiel (CAU) hat die Fütterung von Nematoden (*Panagrolaimus* sp. NFS 24-5) mit *C. cohnii* getestet und die Methoden zur Herstellung angereicherter Nematoden weiterentwickelt. *C. cohnii* wurde dabei jeweils mit S.presso verglichen, einem kommerziellen DHA Anreichungsprodukt für Lebendfutter in der Aquakultur. Es konnte gezeigt werden, dass nur ein geringer Anteil intakter *C. cohnii* Zellen von den Nematoden aufgenommen werden kann. Dadurch wurde ein sehr hoher Algenanteil im Futter benötigt und dennoch war es nicht möglich, ähnliche gute Anreicherungserfolge zu erzielen wie mit S.presso. Werden die Algenzellen allerdings vor der Verfütterung aufgeschlossen, können sogar bessere Ergebnisse erzielt werden als mit S.presso (DHA/mg Trockengewicht der Nematoden: *S.presso* Fütterung 3 mg, *C. cohnii* Fütterung 6,6 mg).

Die CAU entwickelte eine Trocknungs- und Lagerungsmethode für die angereicherten Nematoden. Vor der Austrocknung der Nematoden bei 53 % relativer Luftfeuchtigkeit müssen die Nematoden zunächst eine Adaptationsphase bei höherer Luftfeuchtigkeit (97 %) durchlaufen. Während dieser Adaptationsphase ging allerdings ein Teil der ungesättigten essentiellen Fettsäuren verloren. Dies konnte weitgehend dadurch verhindert werden, indem die Nematoden während dieser Zeit auf 4°C heruntergekühlt wurden und anschließend kühl gelagert wurden.

Schließlich wurden zweimal mehrere Versuchsreihen mit jeweils 5-6 Wiederholungen im Nationalen Polytechnischen Institutes in La Paz (Centro de Investigaciones Biológicas del Noroeste S. C., Mexiko) durchgeführt, da innerhalb von Deutschland oder Europa keine Shrimplarven zur Verfügung gestellt werden konnten. Bei den ersten Versuchsreihen reichte die verwendete Nematodendichte nicht aus, um ähnlich gute Ergebnisse hinsichtlich des Wachstums wie bei der Fütterung mit Artemien zu erzielen. Es konnte aber gezeigt werden, dass die Shrimplarven die angereicherten Nematoden fressen. PL1-Larven, die ausschließlich mit angereicherten Nematoden gefüttert wurden, hatten einen mehr als doppelt so hohen DHA-Gehalt als die Larven, die mit Artemien gefüttert wurden. Die Nematodendichte wurde im 2. Versuch erhöht und außerdem wurde der Fütterungszeitpunkt für die Fütterung mit Nematoden um 2 Tage vorverlegt. Artemienlarven sind zu dieser Zeit noch zu groß für die Shrimplarven, während Nematoden auf Grund ihrer geringeren Größe schon angenommen werden. In der Folge konnten hinsichtlich Wachstum, Überlebensrate und DHA-Gehalt der Shrimplarven gleich gute oder bessere Ergebnisse als bei der Fütterung mit Artemien erzielt werden. Bis zum Mysis I Stadium wurde mit der Fütterung von Nematoden signifikant besser Ergebnisse erzielt als mit Artemien, selbst mit vorher nicht angereicherten Nematoden. Aufgrund der relativ geringen Aufwandmengen an Nematoden und der einfacheren Anwendung wäre die Verwendung von Nematoden bis zum Mysis I Stadium auf jeden Fall ökonomischer als die Verwendung von Artemien. Bei der weiteren Fütterung bis zum PL1 und PL6 Stadium war die Fütterung mit Artemien und angereicherten Nematoden gleichwertig. Allerdings müssen zunehmend höhere Nematodendichten angewendet werden. Weiterhin werden normalerweise ab dem PL1 Stadium neben Artemien verschiedene kostengünstige Trockenfutterbestandteile verwendet und keine reine Artemienfütterung mehr durchgeführt, wie in den durchgeführten Versuchen. Ob eine Fütterung mit Nematoden ab dem Mysis I Stadium eine ökonomisch sinnvolle Variante darstellt, muss deshalb noch in weiteren Praxisversuchen getestet werden.

Aufgrund der im Projekt erzielten Ergebnisse ist die Vermarktung von Nematoden für die Aquakultur von Shrimps sehr erfolgversprechend. Aus diesem Grund entwickelt die e-nema GmbH nach Abschluss des DBU Projektes die Flüssigkultur der Nematoden und ihre Anreicherung und Trocknung im Industriemaßstab weiter. Weitere Praxisversuche in Zusammenarbeit mit dem Nationalen Polytechnischen Institutes in La Paz werden im Frühjahr 2015 durchgeführt.

1 Projektpartner E-NEMA GmbH

1.1 Arbeitsplan laut Antrag

Wesentliches Ziel war die Entwicklung eines zweistufigen Fermentationsverfahrens für heterotrophe Algen zur Optimierung der DHA-Ausbeute. Als erste Grundlage wurden veröffentlichte Protokolle für *Crypthecodinium cohnii* (Swaaf et al. 2003) und *Schizochytrium* sp. genutzt (Ganuza et al. 2008).

Algenkultur: Schüttelkolbenversuche	
Optimierung der Zellzahl	☑
Optimierung der DHA-Ausbeute	☑
Algenkultur: Fermentationen	
Übertragung optimiertes Fermentations-Protokoll in 10 und 20 l Stahlfermenter	☑
Scale-up in den Pilotmaßstab (500 l)	☑
Algenaufbereitung	
Entwicklung der Parameter & Bereitstellung von Algen für die Anreicherung von Nematoden	☑

1.2 Fermentation von *Crypthecodinium cohnii*

1.2.1 Schüttelkolbenversuche

Der von Swaaf et al. (2003) beschriebene Prozess wurde an die Gegebenheiten der e-nema GmbH angepasst. Zur Fütterung wurde Ethanol eingesetzt, weil (1) die Ausbeute an DHA höher ist (53 mg l^{-1} h^{-1}) als mit Essigsäure (48 mg l^{-1} h^{-1}) oder Glucose und (2) Ethanol im Gegensatz zu Essigsäure nicht korrosiv ist. Ethanol ist außerdem günstiger als Essigsäure. Zur Regelung des pH-Werts wurde die weniger korrosive Phosphorsäure statt der bei de Swaaf et al. (2003) verwendeten Salzsäure eingesetzt.

1.2.2 Fermentationen

Fermentationen wurden in 10 und 20 l Edelstahlfermenter erfolgreich durchgeführt (Abb. 1-3). Es wurden ähnliche Mengen DHA produziert wie in den besten in der Literatur beschriebenen Prozessen (de Swaaf et al. 2003).

Ein automatischer Fütterungsmodus für die Kultivierung von *C. cohnii* wurde entwickelt, aber die Einstellung für den Bioreaktor erwies sich als schwierig und ergab unvorhersehbare Erträge. Aus diesem Grund haben wir uns im weiteren Verlauf für eine manuelle Fütterungsmethode während des Prozesses entschieden.

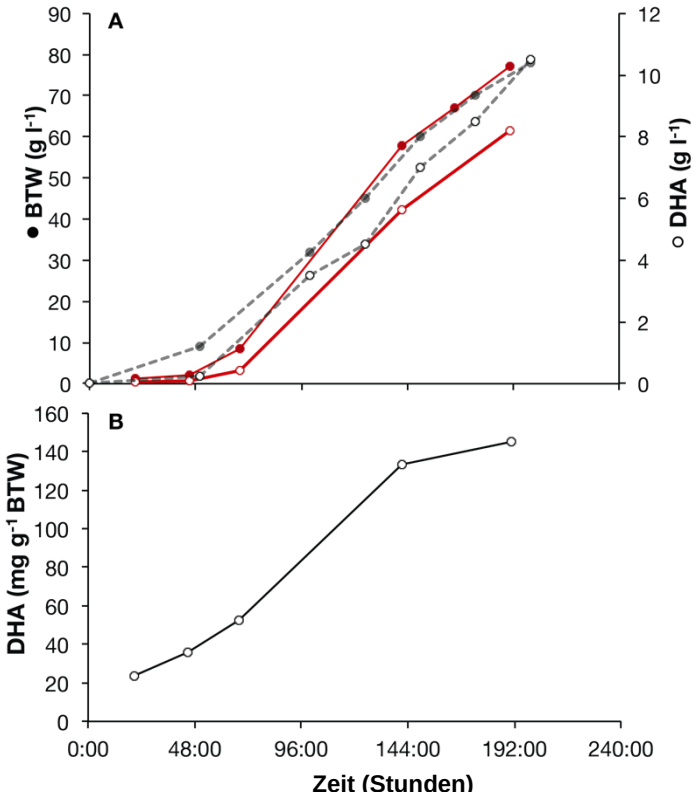

Abb. 1 Docosahexaensäure (DHA)-Gehalt (**A** in g l^{-1} and **B** in mg g^{-1} BTW) in Kulturen von *Crypthecodinium cohnii* im 10-l Bioreaktor, mit Ethanol als C-Quelle. Die gestrichelten Linien geben die besten in der Literatur beschriebenen Ergebnisse wieder (de Swaaf et al., 2003).

Die Biotrockenmasse (BTW) erwies sich als ein guter Indikator zur Abschätzung der DHA Produktion während der Fermentation (Abb. 2). Je höher die BTW [g l^{-1}] desto höher war auch der spezifische DHA Gehalt in der Biotrockenmasse. Fehlgeschlagene Läufe konnten so frühzeitig erkannt werden und die Prozessoptimierung beschleunigt werden.

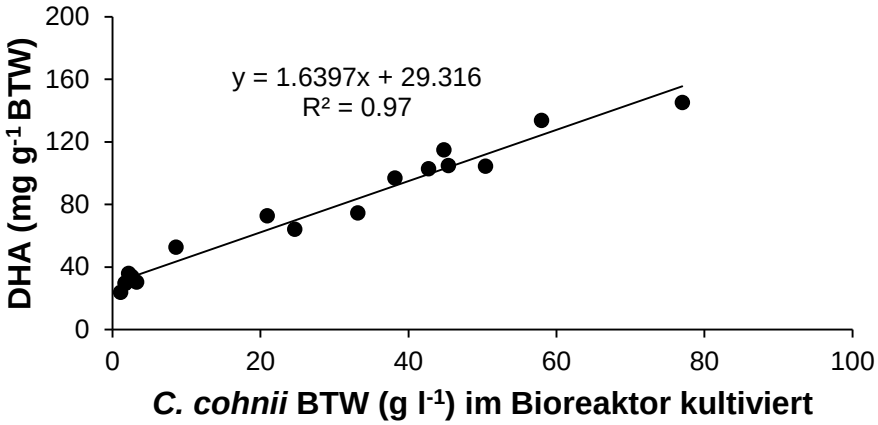

Abb. 2 Beziehung des DHA-Gehalts in der Trockenmasse von *C. cohnii* und der Konzentration der Biotrockenmasse der Alge im Kulturmedium (n=8).

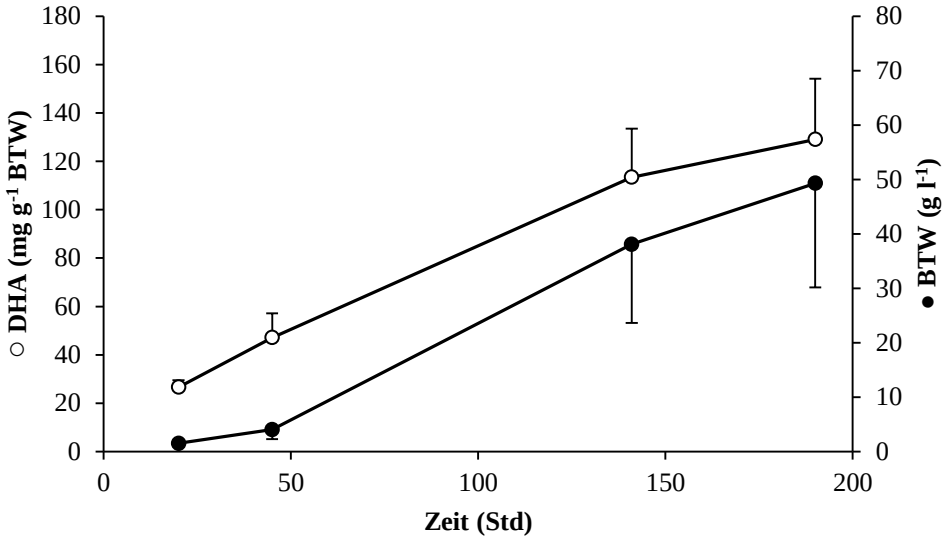

Abb. 3 Mittlere Docosahexaensäure (DHA) Konzentration (weiße Kreise) and Trockenbiomasse (schwarze Kreise) ± SD bei Kultivierung des Dinoflagellaten *Crypthecodinium cohnii* (ATCC 30772) in 10- und 20 l Bioreaktoren (*n*=7) mit Ethanol als C-Quelle.

1.2.3 Produktion im 500-l Bioreaktor

Es wurden 2 Bioreaktorläufe im 500 l Maßstab durchgeführt. Im ersten Lauf wurden aufgrund von Störungen in der automatischen Fütterung der Algen keine zufriedenstellenden Zelldichten und Biotrockenmassewerte erzielt (Abb. 4).

Der im 2. Lauf erzielte Ertrag an DHA (4.1 g l^{-1}, Abb. 5) war vergleichbar mit Ratledge et al. (2001) im 5 l (4.4 g l^{-1}) und de Swaaf (2003) im 150 l Bioreaktor (4.6 g l^{-1}) mit dem Stamm (ATCC 30772) erzielten Ertrag (Tab. 1). Dennoch war der beobachtete DHA-Gehalt nur etwa halb so hoch wie der in den Laborfermentern erzielte (bis zu 8 g l^{-1}) oder den publizierten Ergebnissen aus 2 l Bioreaktoren bei Verwendung von Ethanol als C-Quelle (10 g l^{-1}) (de Swaaf et al. 2003). Die beobachtet Diskrepanz liegt wahrscheinlich an den im größeren Maßstab veränderten Bedingungen (z.B. andere Rührwerksgeometire und –drehzahl, andere Druckverhältnisse und CO_2 Konzentrationen etc.).

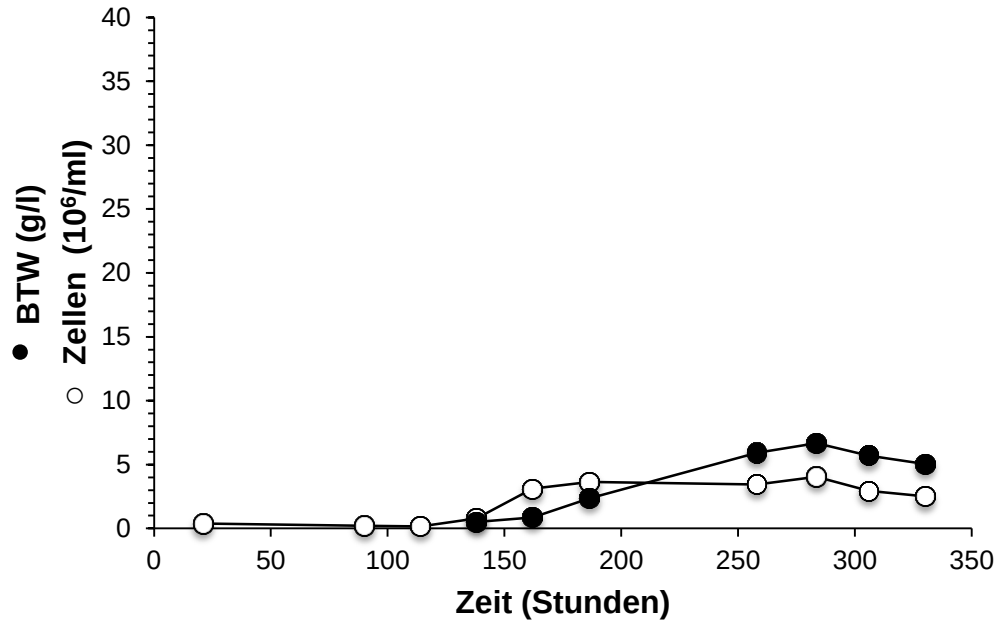

Abb. 4 Zelldichten und Biotrockenmasse des Dinoflagellaten *Crypthecodinium cohnii* im 500-l Bioreaktor (1. Versuch) mit Ethanol als C-Quelle.

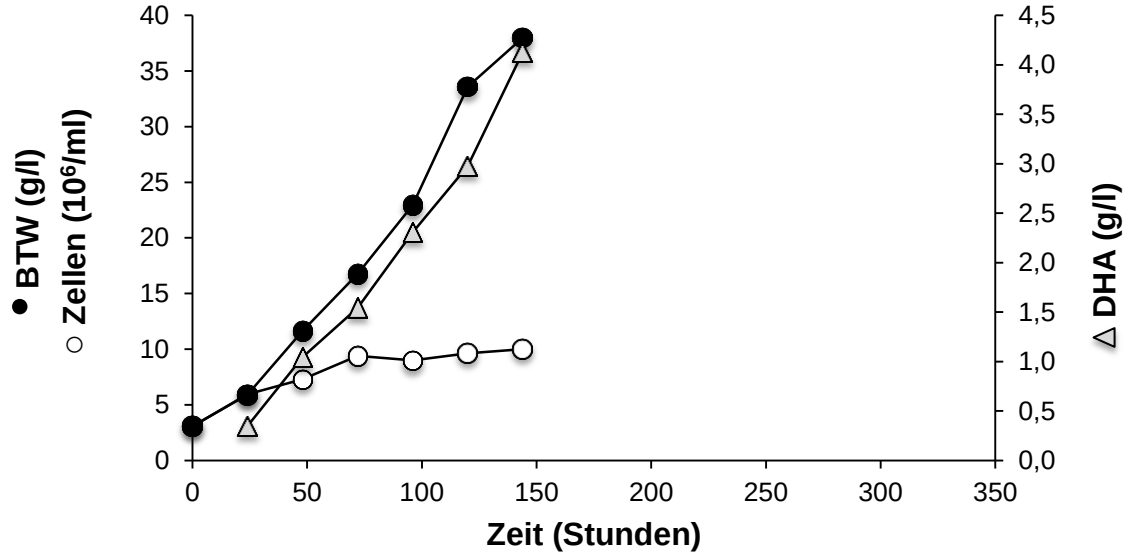

Abb. 5 Zelldichte, Biotrockenmasse und DHA-Produktion von *Crypthecodininium cohnii* im 500-l Bioreaktor (2. Versuch) mit Ethanol als C-Quelle.

Tab. 1 Vergleich der Lipid und Fettsäuregehalte von *Crypthecodinium cohnii* aus unserer Produktion (500 l Bioreaktor) mit Werten aus der Literatur (150 l Bioreaktor) nach de Swaaf et al. (2003).

	Bioreactor	
	150 l	500 l
Zeit (Std.)	136	144
Biomasse (g l^{-1})	33	38
Lipid-Gehalt (%w/w)	49	32
DHA Anteil an Lipiden (%w/w)	28	34
DHA Konzentration (g l^{-1})	4.6	4.1
r_{DHA} (mg l^{-1} h^{-1})	34	29
Substrat l^{-1}	178	116

1.2.4 Aufarbeitung der Algen

Um die Verfügbarkeit der DHA für die Nematoden zu verbessern wurde der Aufschluss der Algenzellen mit einem Hochdruck-Homogenisator (bei 1000 bis 1500 bar) getestet. Nach Hochdruck-Homogenisation betrug die Größe der kleinsten Partikel 1 bis 5 µm (Abb. 5), was in etwa der Partikelgröße in dem kommerziellen DHA-Anreicherungsprodukt S.presso® entspricht (INVE, Belgien). Diese Partikelgröße entspricht zudem der Mundöffnung (4.2 µm) von adulten Nematoden (*Panagrolaimus* sp., NFS-24-5) und führt daher zu einer effizienteren Nutzung des Anreicherungsprodukts durch den Nematoden, wie von unserem Projektpartner gezeigt wurde (s. u.). Der Hochdruck-Homogenisator kann hochkonzentrierte Algensuspensionen von bis zu 120 g dw l^{-1}) schnell (> 20 l h^{-1}) aufschließen. Die Temperatur kann dabei über einen Wärmetauscher niedrig gehalten werden. Unser Projektpartner hatte für den Zellaufschluss zunächst mit Flüssigstickstoff tiefgekühlte Algen verwendet; ein Verfahren was weniger effizient ist (Abb. 6) und für die industrielle Produktion ungeeignet wäre. Der Hochdruck-Homogenisator wäre eine geeignete Alternative.

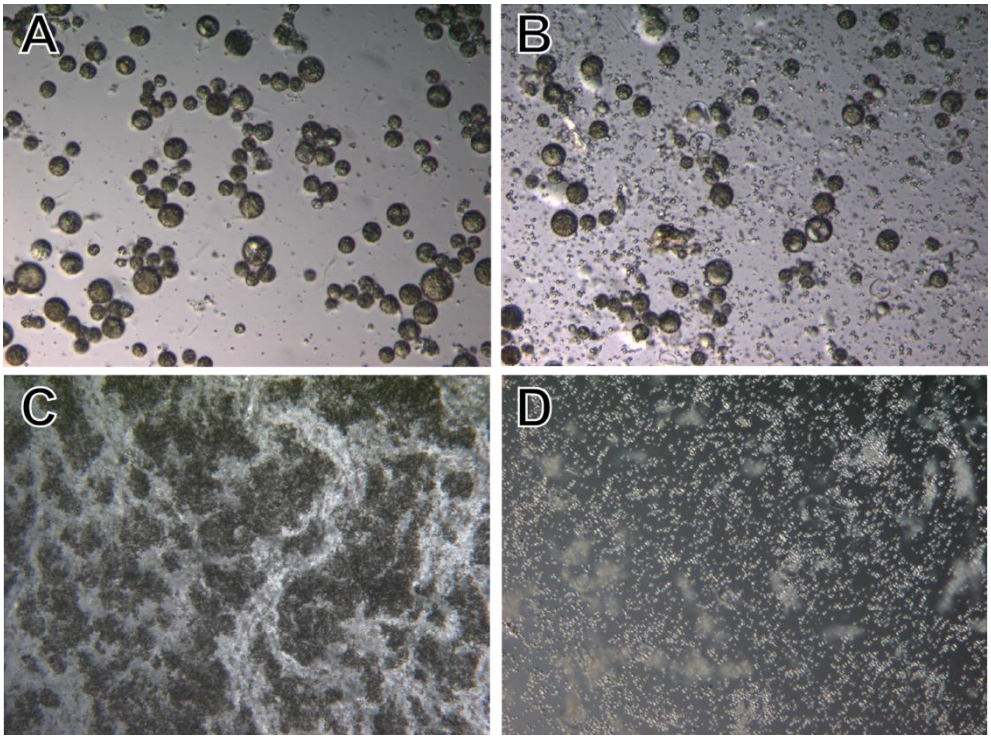

Abb. 6 *Crypthecodinium-cohnii*-Zellen (10-fach vergrößert) vor Aufschluss (A), nach 1 min mahlen in Flüssigstickstoff (B), und nach Homogenization mit einem Hochdruck-Homogenisator (EmulsiFlex-D20 Avestin, Canada) bei 500 bar. (C). Bild D zeigt das Material aus (C) bei 100-facher Vergrößerung.

Nach Homogenisation wurde dem Algenbrei 0.01% (w/v) des Nahrungsmittel-Antioxidants butyliertes Hydroxytoluen beigemengt. Die homogenisierten *C. cohnii* können bei -20°C gelagert werden.

Es wurden mehrere Verfahren getestet, um die Algen nach der Ernte so zu lagern, dass eine Oxidation der wertvollen ungesättigten Lipide verhindert wird. In Tabelle 2 wird die Qualität von gefrier- und sprühgetrocknetem Material verglichen. Die Sprühtrocknung wurde bei 180°C Eingangstemperatur und 90°C Austrittstemperatur betreiben. Die Algensuspension wurde dabei durch eine rotierende Scheibe als feiner Nebel in den Sprühturm gegeben. Der Lipid-Gehalt und die Fettsäurezusammen-setzung waren bei beiden Trocknungsmethoden ähnlich. Die Sprühtrocknung ist bei einer Maßstabsvergrößerung die bevorzugte Methode, da sie weit günstiger ist als die Gefriertrocknung.

Tab. 2 Vergleich des Lipidgehalts und der Fettsäurezusammensetzung von *Crypthecodinium cohnii* produziert im 500-l Bioreaktor nach Trocknung im Gefriertrockner und im Sprühtrockner.

	Gefriergetrocknet	**Sprühgetrocknet**
Lipidgehalt (% w/w)	26.3 ± 0.3	26.0 ± 0.7
davon	% w/w	% w/w
12:0	7.5 ± 0.0	7.1 ± 0.3
14:0	14.9 ± 0.0	14.0 ± 0.5
16:0	13.4 ± 0.0	12.6 ± 0.5
16:1	1.9 ± 0.0	1.7 ± 0.1
18:0	0.3 ± 0.0	0.3 ± 0
18:1	8.3 ± 0.0	7.8 ± 0.2
DHA	37.7 ± 0.1	35.0 ± 1.6

1.2.5 Maßstabsvergrößerung der Nematodenproduktion und Energieverbrauchsdiagnose

Es sei an dieser Stelle betont, dass dieser Schritt nicht Bestandteil des Projektantrags war. Um jedoch Fütterungsversuche in größeren Maßstab durchzuführen zu können, musste eine größere Menge Nematoden produziert werden. Dazu wurde die Produktion von *Panagrolaimus* sp. optimiert (Ayub et al. 2013-2014, siehe Anhang) und ein scale-up der Produktion auf Pilotmaßstab (500 l) durchgeführt. Die Nematoden erreichten im 500 l Bioreaktor eine Dichte von 89.000 ml^{-1}, was bisher deutlich unter den Ausbeuten im Laborfermenter mit 336.000 ml^{-1} im Mittel lag (n=12, Fermentervolumen 5-30 l). Die höchste erzielte Nematodendichte im Bioreaktor betrug 875.000 ml^{-1}. Der Anreicherungsprozess mit aufgeschlossenen *C. cohnii* und mit S.presso® und der Austrocknungsprozess wurde ebenfalls im Pilotmaßstab durchgeführt. Die Nematoden wurden dann bei 4°C und 53% rel. Luftfeuchtigkeit gelagert. Über einen Lagerungszeitraum von 5 Monaten zeigte das Fettsäureprofil und die Überlebensrate der Nematoden keine Einbußen. Für die Trocknung und Lagerung der angereicherten Nematoden muss allerdings erst noch ein für den Industriemaßstab geeignetes Verfahren entwickelt werden.

Schließlich wurden dem ECO-Institut GmbH Daten zum Energieverbrauch und zum Verbrauch chemischer Grundstoffe für die Produktion angereicherter Nematoden im Großmaßstab zur Verfügung gestellt.

2 Projektpartner Christian-Albrechts-Universität zu Kiel

2.1 Arbeitsplan laut Antrag

Ziel war es Shrimplarven erfolgreich mit Nematoden zu füttern und damit eine mögliche Alternative für die Fütterung von Artemia-Larven zu finden. Artemien sind ebenfalls Krebstiere und können Krankheiten auf Shrimps übertragen. Shrimplarven sind sehr anspruchsvoll hinsichtlich des DHA/EPA-Gehalts und Mengenverhältnisses (mindestens 2:1) im Futter. Aus diesem Grund musste zunächst eine Möglichkeit gefunden werden, auch die Nematoden mit den entsprechenden Fettsäuren anzureichen. Als Quelle für DHA sollten dabei Algen verwendet werde.

Anreicherung von Nematoden	
DHA-Anreicherung mit heterotrophen Algen aus Schüttelkolben	☑
Optimierung der DHA/EPA Relation mit heterotrophen Algen (und Anreicherung mit S.Presso von INVE)	☑
DHA-Anreicherung mit trockneten Algenkulturen	☑
Fettsäureanalytik mit GC	☑
Fütterungsversuche Shrimps mit angereicherten Nematoden	
Fütterung von *L. vannamei* im Labormaßstab, max 2 l	☑
Fütterung von *L. vannamei* im Pilotmaßstab, 50-200 l	☑

2.2 Anreicherung von Nematoden mit DHA

Eine vorangehende Studie hatte gezeigt, dass n-3 HUFA Fettsäuren (insbesondere DHA und EPA) in Nematoden angereichert werden können und dabei 6-7-mal höhere Trockenmassenanteile erreicht werden können, als in Rotiferen. Die Nematoden wurden dazu mit Lebertran (10 %) angereichertem Medium gefüttert (Reyes et al. 2011). Wir testeten im Vergleich dazu ein Medium, dass mit 10, 20 und 30 % *C. cohnii* Algen in der Trockenmasse des Mediums (Tabelle 2) angereichert war und konnten dabei höhere DHA-Anteile und damit ein besseres DHA/EPA-Verhältnis in den Nematoden erzeugen, als es mit Lebertran erzielt wurde. Aufgrund des hohen Anteils an Algen im Futter der Nematoden wäre diese Methode allerdings unökonomisch. Die Fettsäureanalysen wurden bei der e-nema GmbH durchgeführt.

Tab. 2 Fettsäuregehalt von Nematoden, die mit *C. cohnii* oder Lebertran gefüttert wurden (Reyes et al. 2011).

	Quelle und Anreicherungsprodukte	
	E-NEMA (2012) *C. cohnii* (20%) w/w Trockengew.	Reyes et al. 2011 Lebertran (10%) w/w Nassgew.
Fettsäuren	% vom Gesamtgehalt	
C20:4n-6 (AA)	7.7 ± 0.4	0.7 ± 0.0
C20:5n-3 (EPA)	3.1 ± 0.3	9.2 ± 0.2
C22:6n-3 (DHA)	15.7 ± 1.9	8.5 ± 0.6
DHA/EPA	5.1	0.9

Die Nematoden können nur einen kleinen Teil der Algen verwerten, da der Durchmesser von *C. cohnii* mit 15-50 µm zu groß ist (Abb. 6). Wir testeten deshalb die Fütterung mit zermahlenen Algenzellen. Die Algen wurden dazu mit Flüssigstickstoff auf -196°C in einer Keramikmühle heruntergekühlt und zermahlen (Zheng et al. 2011). Die niedrige Temperatur verhindert dabei die Oxidation der ungesättigten Fettsäuren. Durch die Fütterung mit den zerkleinerten *C. cohnii* konnte der DHA-Gehalt der Nematoden um das 3-fache gesteigert werden (Abb. 7).

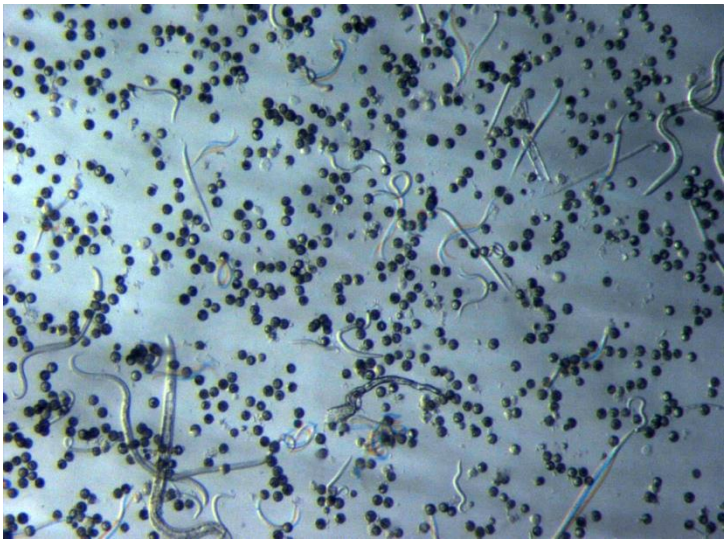

Abb. 6 Darstellung der Größenverhältnisse von *Panagrolaimus* sp. und intakten Zellen von *Crypthecodinium cohnii*.

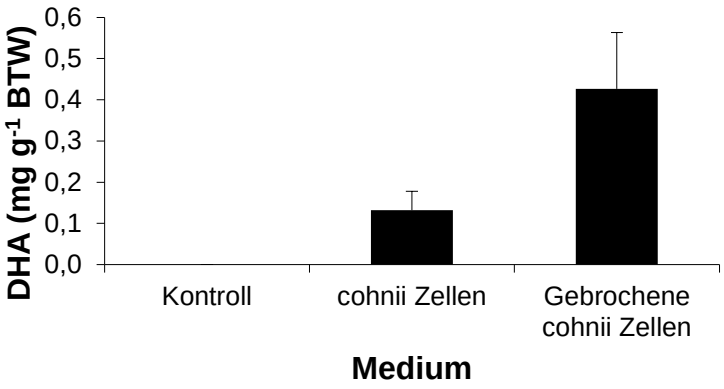

Abb. 7 DHA-Gehalt von Nematoden, die mit intakten oder zermahlenen *C. cohnii*-Zellen gefüttert wurden.

Dennoch konnten auch bei der Fütterung von aufgeschlossenen Zellen keine so hohen DHA-Werte (5.2 ± 2.0 % TFA) in den Nematoden erzielt werden, wie bei der Fütterung mit dem kommerziellen Anreichungsprodukt S.presso (8.7 ± 0.2 % TFA), welches auch für die Anreichung von Artemien verwendet wird (Tabelle 3).

Tab. 3 Fettsäurezusammensetzung (Anteil an TFA in Prozent) bei unterschiedlicher Fütterung:

Ohne Zugabe von DHA zum Futter (nicht angereichert), Zugabe von 1% (w/v) *Crypthecodinium cohnii* (DHA: 144.9 ± 9.8 mg g^{-1} dw) oder Zugabe von 1% (w/v) S.presso (DHA: 135 mg g^{-1} dw) (INVE, Belgien). Unterschiedliche Buchstaben neben den Prozentangaben zeigen signifikante Unterschiede (P<0.05).

SFA: Gesättigte Fettsäuren, MUFA: Einfach ungesättigte Fettsäuren, PUFA: Mehrfach ungesättigte Fettsäuren.

Fettsäuren	nicht angereichert	*C. cohnii*	S.presso
	Anteil an Fettsäuren gesamt (TFA %)		
Σ SFA	15.8 ± 8.3a	31.7 ± 3.2b	17.8 ± 0.3a
Σ MUFA	47.5 ± 13.1b	26.0 ± 4.1a	39.6 ± 0.7b
Σ PUFA	36.6 ± 5.9a	42.3 ± 4.3ab	42.6 ± 0.5b
DHA/EPA	0.0 ± 0.0a	0.8 ± 0.2b	1.1 ± 0.0c
n-3/n-6	0.2 ± 0.1a	0.6 ± 0.3b	0.9 ± 0.0b
	∑(Fettsäureanteil x Anzahl der Doppelbindungen)		
Unsaturation Index	152.1 ± 16.1a	183.3 ± 9.6b	196.0 ± 1.9c

Durch die Verwendung des von unserem Projektpartner getesteten Hochdruck-Homogenisators konnte der Zellaufschluss verbessert werden und dadurch schließlich höhere DHA-Gehalte in den Nematoden bei der Fütterung erzielt werden als mit S.presso (Tabelle 4).

Tab. 4 Fettsäurezusammensetzung (Anteil an TFA in Prozent) bei unterschiedlicher Fütterung:

Ohne Zugabe von DHA zum Futter (nicht angereichert), Zugabe von 1% (w/v) *Crypthecodinium cohnii* (DHA: 114 mg g^{-1} dw) oder Zugabe von 1% (w/v) S.presso (DHA: 135 mg g^{-1} dw) (INVE inc.).

	C. cohnii	S.presso	nicht angereichert
	Anteil an Fettsäuren gesamt (TFA %)		
Σ SFA	20.2 ± 0.6	19.6 ± 0.2	16.8 ± 0.2
Σ MUFA	33.2 ± 1.3	32.1 ± 0.1	34.0 ± 0.3
Σ PUFA	46.6 ± 0.7	48.3 ± 0.1	49.2 ± 0.1
C20:4n-6 (ARA)	8.8 ± 0.1	9.3 ± 0.2	12.4 ± 0.1
C20:5n-3 (EPA)	6.3 ± 0.4	6.3 ± 0.1	4.7 ± 0.1
C22:6n-3 (DHA)	6.7 ± 1.1	4.7 ± 0.3	0.0 ± 0.0
DHA mg g^{-1} BTW	6.6 ± 0.9	3.0 ± 0.4	0.0 ± 0.0
Gesamtfettsäuren (mg g^{-1} BTW)	98.7 ± 3.0	64.0 ± 5.1	75.2 ± 2.4
DHA/EPA	1.1 ± 0.1	0.8 ± 0.0	0.0 ± 0.0
	∑(Fettsäureanteil x Anzahl der Doppelbindungen)		
U.I.	197.3 ± 6.0	194.7 ± 0.8	183.3 ± 0.7

2.3 Trocknung und Lagerung von angereicherten Nematoden

Nach der Anreicherung der Nematoden muss verhindert werden, dass die essentiellen Fettsäuren oxidieren oder von den Nematoden metabolisiert werden, bevor sie an die Shrimplarven verfüttert werden. *Panagrolaimus* ist zur Anhydrobiose fähig, d. h. bei Austrocknung der Nematoden wird deren Stoffwechsel heruntergefahren was zumindest die Metabolisierung erheblich mindern könnte. Im ausgetrockneten Zustand können die Nematoden lange Zeit gelagert werden. Die Stabilität der Fettsäuren während der Lagerung bei 25°C und 4°C wurde untersucht. Es zeigte sich, dass trotz Anhydrobiose der Anteil der ungesättigten Fettsäuren während der Lagerung kontinuierlich zurückgeht (Abb. 8-9), wobei nach einem Monat der Verlust von DHA bei 4°C (11%) deutlich geringer war als bei 25°C (60%) (Abb. 9).

Die Abnahme von DHA in den angereicherten Nematoden war jeweils zu Beginn der Lagerung am höchsten. Dieser Verlust konnte durch Kühlung auf 4°C vor der Austrocknung deutlich reduziert (Abb. 10). Der Austrocknungsprozess erfolgt in zwei Stufen. Zunächst werden die Nematoden einer Luftfeuchtigkeit von 97% ausgesetzt. Durch diese Konditionierungsphase werden in den Nematoden physiologische Prozesse in Gang gesetzt, die die Zellen vor Schäden bei stärkerer Austrocknung schützen. Die weitere Austrocknung erfolgt dann bei 53% relativer Luftfeuchtigkeit.

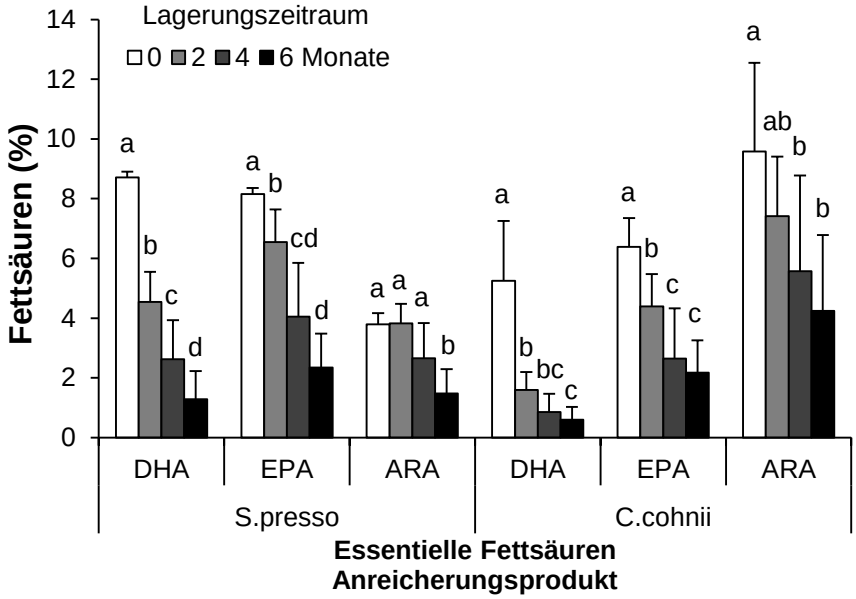

Abb. 8 Relativer Gehalt an essentiellen Fettsäuren (Mittelwert ± SD) in Nematoden während der Lagerung bei 25°C. Verschiedene Buschstaben über den Säulen zeigen signifikante Unterschiede während der Lagerung für eine Fettsäure an (P<0.05).

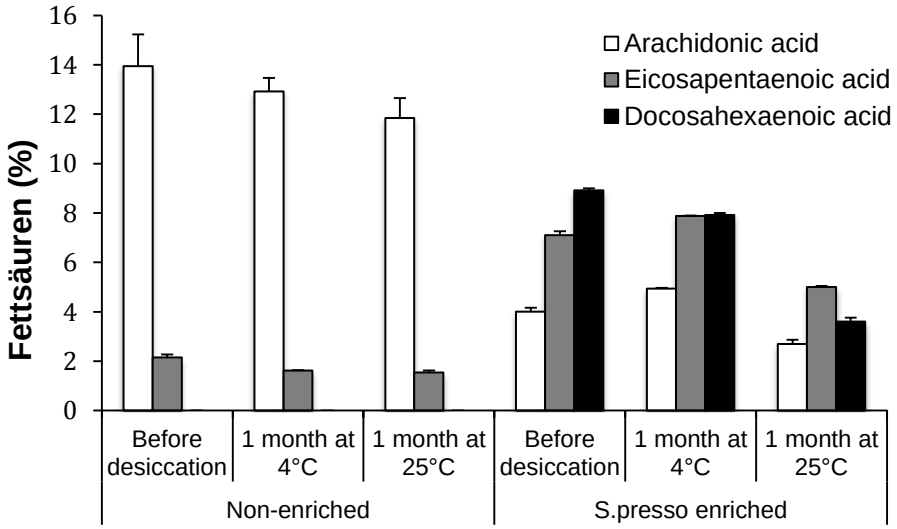

Abb. 9 Relativer Gehalt an essentiellen Fettsäuren (Mittelwert ± SD) in Nematoden während der Lagerung bei 4 und 25°C.

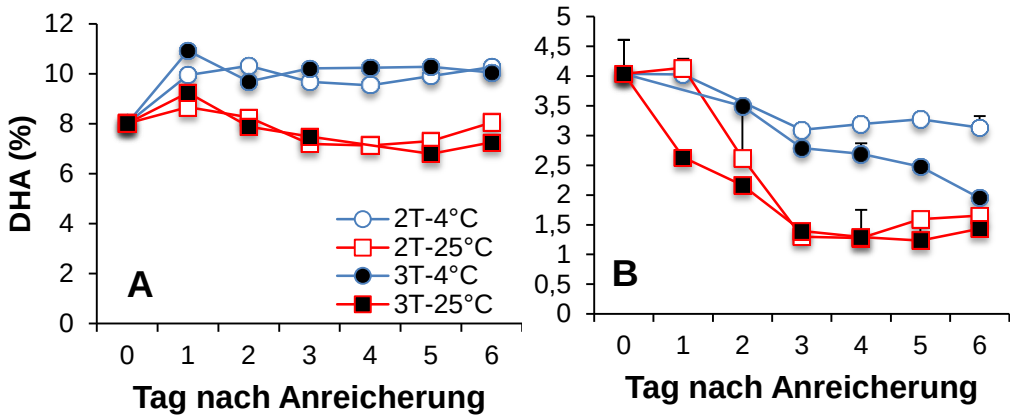

Abb. 10 Relativer Gehalt von DHA an den gesamten Fettsäuren (Mittelwert ± SD) in Nematoden während Austrocknung bei 4° und 25 °C. 2T steht für 2 Tage Konditionierung bei 97% rel. Luftfeuchtigkeit und 3T für 3 Tage.
A) Anreicherung mit 1% S.presso. **B**) Anreicherung mit 0,5% S.presso.

2.4 Fütterung von Shrimplarven mit angereicherten Nematoden

Die Versuche mit Shrimplarven wurden von Dr. Seychelles in den Zuchtanlagen des Nationalen Polytechnischen Institutes in La Paz (Centro de Investigaciones Biológicas del Noroeste S. C., Mexiko) durchgeführt, da innerhalb von Deutschland oder Europa keine Shrimplarven zur Verfügung gestellt werden konnten. Die getrockneten Nematoden wurden von der e-nema GmbH in Plastikbeuteln mit Stickstoffatmosphäre zur Verfügung gestellt. Die Stickstoffatmosphäre verhindert die Oxidation der ungesättigten Fettsäuren während der Lagerung und des Transportes.

2.4.1 Versuch 1-2

2.4.1.1 Versuchsdesign

Die Versuche wurden in belüfteten 2 l Plastikflaschen mit 1,5 l Meerwasser und 100 *Litopenaeus vannamei* Nauplien l^{-1} durchgeführt (Abb. 11). Ab dem Zoea 2 Stadium wurden die *L. vannamei*-Larven mit angereichten Nematoden, frisch geschlüpften Artemialarven oder einer Mischung von beidem gefüttert. Bei jeder Versuchsvariante wurde solange gefüttert, bis 80% das postlarvale Stadium PL1 erreicht hatten. Das Versuchsdesign für Versuch 1 ist in Abb. 12 und Tabelle 5 dargestellt. Im Versuch 2 wurden nur die Varianten A, 50A, NC und NS getestet (siehe Abb. 12).

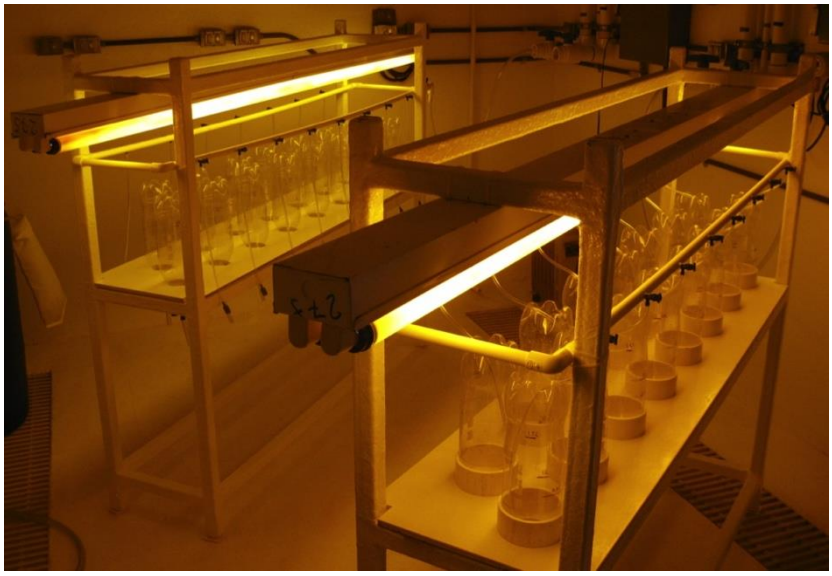

Abb. 11 Belüftete und beleuchtete Plastiktanks für Fütterungsversuche mit *Litopenaeus vannamei* Larven. Der Tag-Nacht-Rhythmus betrug jeweils 12 Stunden.

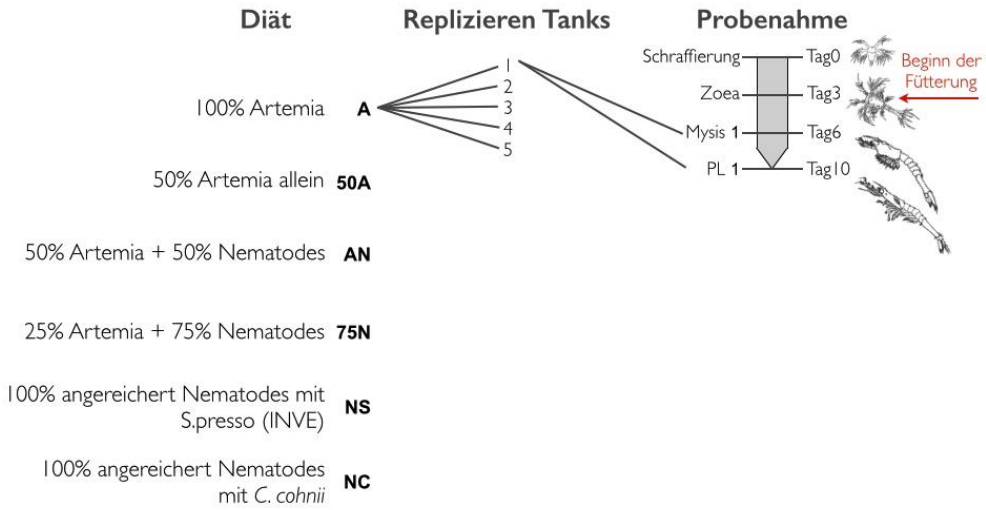

Abb. 12 Versuchsdesign für Versuch 1 mit *Litopenaeus vannamei* Larven mit angereicherten Nematoden *Panagrolaimus* sp. (NFS 24-5) und Artemialarven. Insgesamt 6 verschiedene Diäten × je 5 Tanks × 2 Probenahmen (nach 4 und 7 Tagen). Mikroalgenfütterung bis Zoea 3 (Zellen/l): A) 150.000, 50A) 75.000, AN) 75.000, 75N) 37.500, NS) und NC) keine Mikroalgen

Tab. 5 Futterdichte (Nematoden bzw. Artemien pro ml) für die verschiedenen Entwicklungsstadien von *Litopenaeus vannamei*. MA: Mikroalgenmischung (70% *C. muelleri* and 30% *I. galbana*) mit 150×10^3 Zellen ml^{-1}.

Bis Stufe	Diät A	50A	AN		75N		NC	NS
Zoea 2	MA	MA/2	MA/2	50	MA/4	75	100	100
Zoea 3	MA	MA/2	MA/2	50	MA/4	75	100	100
Mysis 1	5	2.5	2.5	125	1.25	188	250	250
Mysis 2	7	3.5	3.5	175	1.75	263	350	350
Mysis 3	10	5	5	250	2.5	375	500	500
PL 1	10	5	5	250	2.5	375	500	500

2.4.1.2 Resultate Shrimpfütterung

Nach 6 Tagen konnte im Versuch 1 keine Unterschiede hinsichtlich der Überlebensrate und des Trockengewichtes der Shrimplarven festgestellt werden. Es zeigten sich jedoch deutliche Unterschiede hinsichtlich der Größe der Larven. Bei den Diäten NS und NC, die ausschließlich Nematoden und keinen Mikroalgenzusatz enthielten, waren die Larven signifikant kleiner (Abb. 13). Der Anteil an Mysis 1 Larven ging hier gegen Null und war damit auch signifikant kleiner als bei den anderen Varianten (> 40%). Auch im Versuch 2 waren die Shrimplarven am 6. Tag bei den NS und NC deutlich kleiner und der Anteil an Mysis 1 Larven geringer, auch wenn die Unterschiede zu den anderen Varianten geringer und nicht immer statistisch signifikant waren (Abb. 14).

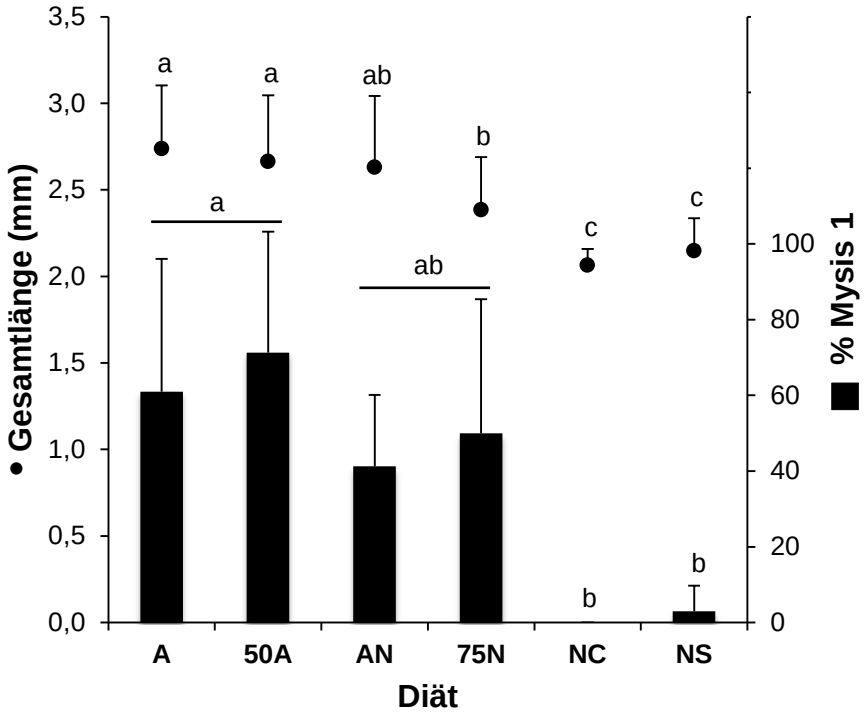

Abb. 13 *Litopenaeus vannamei* Larven Länge und Mysis 1 Anteil (Mittelwert ± SD) nach 6 Tagen bei verschiedenen Diäten (siehe Tabelle 5) im Versuch 1. Verschiedene Buschstaben über den Säulen zeigen signifikante Unterschiede an (P<0.05).

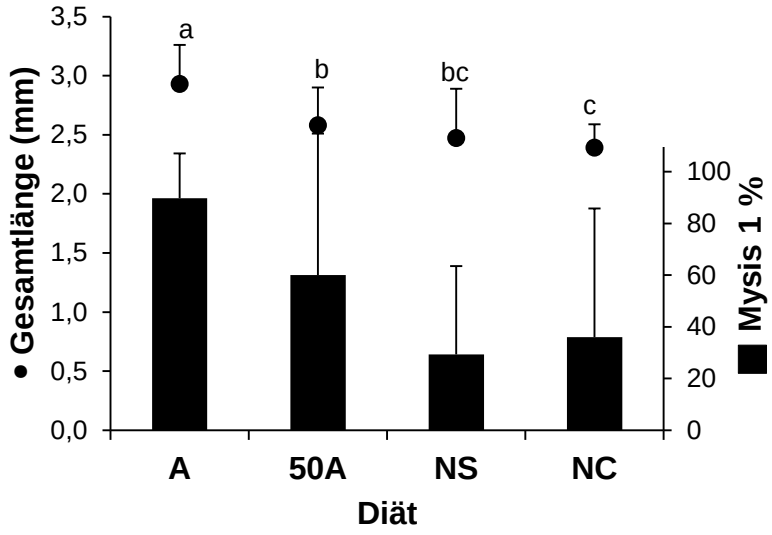

Abb. 14 *Litopenaeus vannamei* Larven Länge und Mysis 1 Anteil (Mittelwert ± SD) nach 6 Tagen bei verschiedenen Diäten (siehe Tabelle 5) im Versuch 2. Verschiedene Buschstaben über den Säulen zeigen signifikante Unterschiede an (P<0.05).

Nach 10 Tagen häuteten sich 90 ± 13% der Larven in den Versuchsvarianten zu PL 1, außer bei der NC Variante mit nur 10% im Mittel (Abb. 15). In den Versuchsvarianten mit 100 und 50 % Artemia-Anteil im Futter (A, 50A) waren die *L. vannamei* signifikant am größten. Die Variante AN, die neben Artemien auch Nematoden enthielt konnte allerdings nicht ausgewertet werden. In den Varianten A, 50A, AN und NS war die Mortalität der Shrimplarven ein Tag vor Probennahme sprunghaft angestiegen und in der Variante AN und NS waren zu wenig Tiere verblieben für die Längenmessung. Die Überlebensrate war in den Varianten 75N und NC am höchsten.

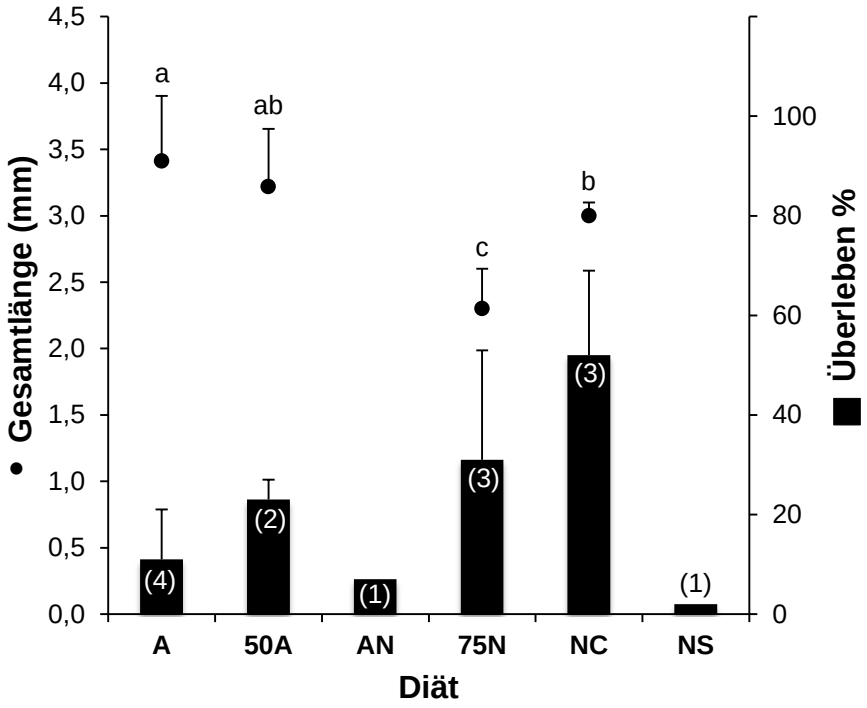

Abb. 15 Länge und Überlebensrate (Mittelwert ± SD) von *Litopenaeus vannamei* nach 10 Tagen (Versuch 1) bei verschiedenen Diäten (siehe Tabelle 5). Zahlen in Klammern geben die Anzahl der auswertbaren Kulturen wieder. Verschiedene Buschstaben über den Datenpunkten zeigen signifikante Unterschiede für die Körperlänge an (P<0.05).

Bei PL1-Larven, die ausschließlich mit angereicherten Nematoden gefüttert wurden (Variante NC) war der DHA-Gehalt doppelt so hoch wie bei Larven, die mit Artemien gefüttert wurden (Abb.: 16). Dies macht auch deutlich, dass die Shrimplarven in der Lage waren, die Nematoden zu verdauen.

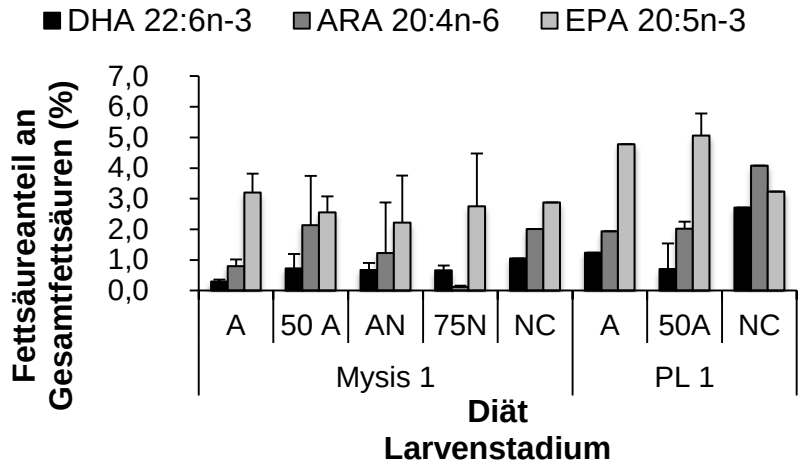

Abb. 16 DHA, ARA und EPA –Gehalt in *Litopenaeus vannamei* Mysis 1 und PL 1 Larven in Abhängigkeit von den Futterorganismen. Siehe Abb.: 12 für Zusammensetzung der verschiedenen Diäten (A, 50A, AN, 75N, NC).

2.4.2 Versuch 3-4

2.4.2.1 Versuchsdesign

Die Versuche wurden in 10-l-Kunststofftanks, mit 9,5 l Meerwasser und 150 Nauplien/l bei 30-31°C durchgeführt. Insgesamt wurden 40.000 Nauplien von 200 Muttertieren verwendet. Im Gegensatz zu den vorangegangenen Fütterungsversuchen wurden die Shrimps im Versuch 4 über das PL1 Stadium hinaus zum PL6 gefüttert. Die Entwicklung von PL5 zu PL6 ist ein kritisches Stadium, da währenddessen die Kiemen ihre volle Funktionalität erlangen.

Das Wachstum der Shrimps wurde für die Fütterung mit *Artemia franciscana* Nauplien sowie angereichten und nicht angereicherten Nematoden verglichen. Bei allen Versuchsvarianten wurden bis zum Mysis 3 Stadium Mikroalgen (MA) zugefüttert (Abb. 18) (70% *Chaetoceros muelleri* (CHAGRA), 30% *Isochrisis galbana* (T-ISO), 150×10^3 Zellen/ml). Die Nematoden konnten aufgrund ihrer geringeren Größe schon 2 Tage vor den Artemienlarven zur Fütterung der Shrimps verwendet werden. Im Versuch 3 wurden 4 verschiedene Diäten getestet und im Versuch 4 wurden die mit *C. cohnii* angereicherten Nematoden (Variante NC) mit S.presso angereicherten Nematoden und Artemien verglichen (siehe Abb. 17-18). Bei der Variante ALG, bei der im Versuch 3 nur Mikroalgen gefüttert wurden, wurde zusätzlich zu der normalen MA-Mischung noch *C. cohnii* gefüttert. Die Biomasse von *C. cohnii* entsprach dabei der Biomasse von *C. muelleri*. Jede Fütterungsvariante wurde im Versuch 3 in 4 und im Versuch 4 in 5 Tanks getestet. Die Fütterungsmenge in den Nematodenvarianten entsprach im Versuch 3 der Biomasse der Artemien und im Versuch 4 dem 1.5 fachen der Artemienmenge (Tabelle 7).

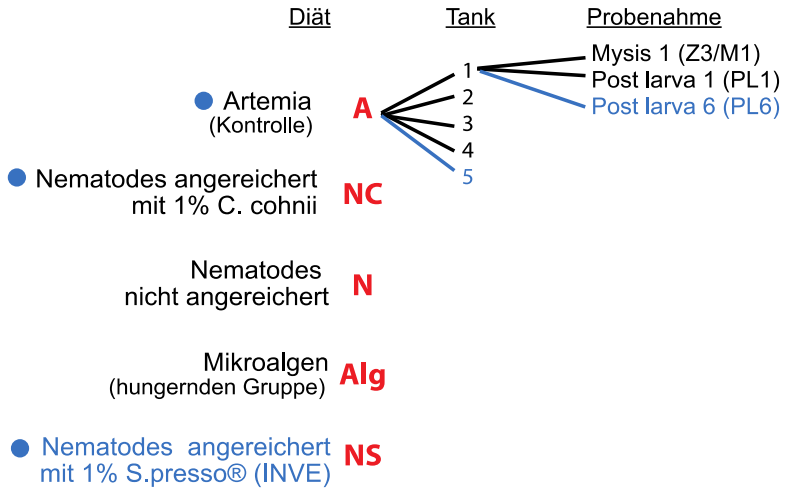

Abb. 17 Versuchsdesign für Fütterungsversuche mit *Litopenaeus vannamei* in 10 l Plastiktanks. Versuch 3: Varianten A, NC, N Alg, Fütterung bis PL1, 4 Tanks je Variante. Versuch 4: Varianten ●, A, NC, NS, Fütterung bis PL6, 5 Tanks je Variante.

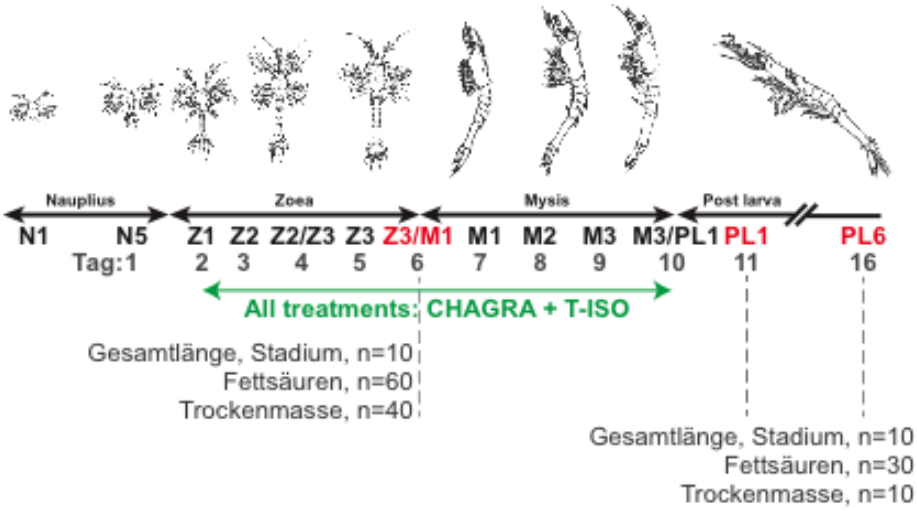

Abb. 18 Probenahmezeitpunkte (rot) und -umfang in den Versuchen 3 und 4.

Tab. 6 Futtermengen pro *L. vannamei*-Larven bei Nematoden und Artemien Diät
Versuch 3: Biomasse von Artemien und Nematoden war äquivalent.
Versuch 4: 1,5-fache Menge Nematoden im Verhältnis zur Biomasse der Artemien

Stage	Versuch 3 und 4 Variante A Artemia nauplii/Larve	Versuch 3 Varianten N, NC Nematoden/Larve	Versuch 4 Varianten NC, NS Nematoden/Larve
Z2		200	300
Z2/Z3		200	300
Z3	3	200	300
Z3/M1	5	317	476
M1	5	302	453
M2	6	398	597
M3	8	514	771
M3/PL1	11	711	1067
PL1	19		1938
PL2	21		2096
PL3	22		2201
PL4	24		2400
PL5	24		2400
PL6	27		2700

2.4.2.2 Resultate

Im Gegensatz zu den Versuchen 1-2 wurde in den Versuchen 3-4 mit der Fütterung mit Nematoden 2 Tage vor der Fütterung mit Artemien begonnen (Tab. 6). Artemien wären zu dieser Zeit zu groß als Futterorganismen. Die früher einsetzende Fütterung mit Nematoden hat sich positiv auf die Entwicklung der Shrimps ausgewirkt. Die Shrimp-Larven waren signifikant größer und die Häutung zum Mysis-1 Stadium häufiger als bei Fütterung mit Artemien (Abb. 19). Dabei machte es keinen signifikanten Unterschied ob oder womit die Nematoden angereichert waren (NC, NS) oder nicht angereichert waren (N). Eine Nematodenzufütterung ab Z2 erscheint deshalb grundsätzlich eine sinnvolle Ergänzung zur Algenfütterung zu sein.

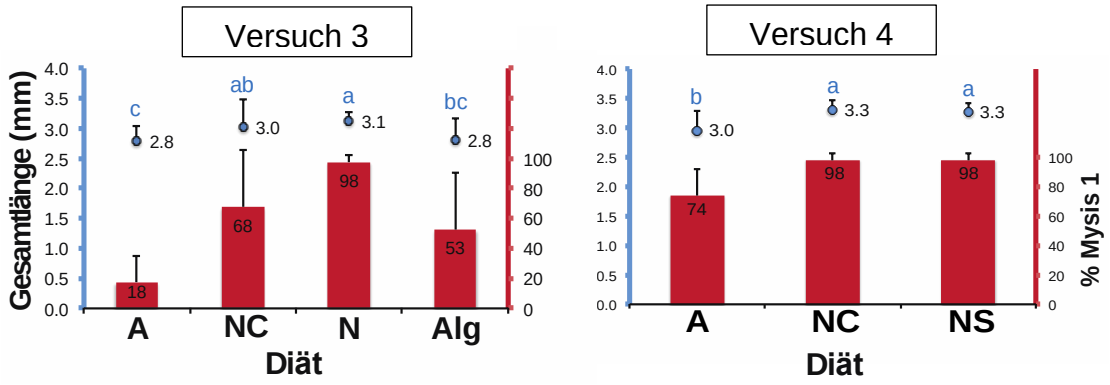

Abb. 19 Länge und Anteil von Mysis-1 Larven in *L. vannamei* Kulturen bei Fütterung mit unterschiedlichen Diäten über 6 Tage bei 30-31°C. Verschiedene Buchstaben über den Datenpunkten zeigen signifikante Unterschiede an (P < 0,05). Zahlen neben den Datenpunkten und in den Säulen geben den erreichten Wert an.

Das Trockengewicht der Mysis-1 Larven zeigte im Versuch 3 keine signifikanten Unterschiede (18,2 ± 3,3 µg pro Individuum, *P*>0.05). Im Versuch 4, bei der die 1.5-fache Menge an Nematoden verfüttert wurden, war das Trockengewicht in der Artemien Variante signifikant kleiner und bei den mit S.presso angereicherten Nematoden (NS) signifikant am größten (Trockengewicht der Schrimplarven, Variante A 14.5 ± 1.1 µg, NC 20,5 ± 2.1 µg, NS 24,0 ± 2,2 µg).

Nach 11 Tagen Fütterung und zunehmenden Größe der Shrimplarven verbesserte sich die Performance der Fütterung mit Artemien gegenüber der Nematodenfütterung (Abb. 20). Im Versuch 3 und 4 lag die Köperlänge der Larven signifikant knapp über der Variante mit angereicherten Nematoden. Der Anteil an PL1 Stadien war im Versuch 3 geringfügig aber signifikant höher und im Versuch 3 gab es diesbezüglich keinen Unterschied. Hinsichtlich des Trockengewichtes je Larve gab es zwischen der Artemien- und den Nematodenvariante keine signifikanten Unterschiede (Versuch 3: 57,7 ± 17,6 µg, Versuch 4: 56,7 ± 28,5 µg). Die Überlebensrate war im Versuch 4 höher (40.0 ± 14.1%) aber nicht signifikant verschieden zum Versuch 3 (29,5 ± 14,2%).

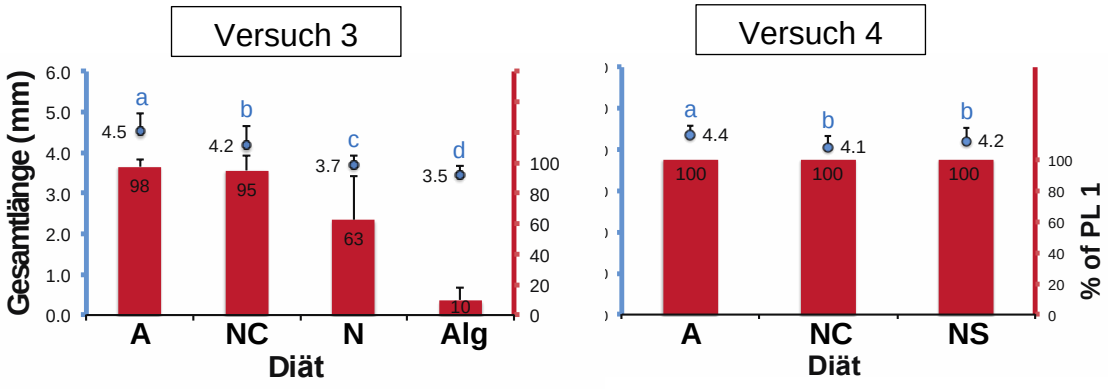

Abb. 20 Länge und Anteil von PL1 Larven in *L. vannamei* Kulturen bei Fütterung mit unterschiedlichen Diäten über 11 Tage bei 30-31°C. Verschiedene Buchstaben über den Datenpunkten zeigen signifikante Unterschiede an (P < 0,05). Zahlen neben den Datenpunkten und in den Säulen geben den erreichten Wert an.

Im Versuch 4 wurde die Fütterung bis zum 16 Tag weitergeführt. In der Variante mit S.presso angereichten Nematoden (NS) verstarben fast alle Shrimplarven zwischen dem 12. und 16. Tag, sodass hierfür keine weiteren Daten erhoben werden konnten. Beim Wachstum der mit Artemien (A) und mit *C. cohnii* angereicherten Nematoden (NC) gab es keine signifikanten Unterschiede (Abb. 21). Auch hinsichtlich der Überlebensrate gab es keine signifikanten Unterschiede (38,7 ± 18,1%).

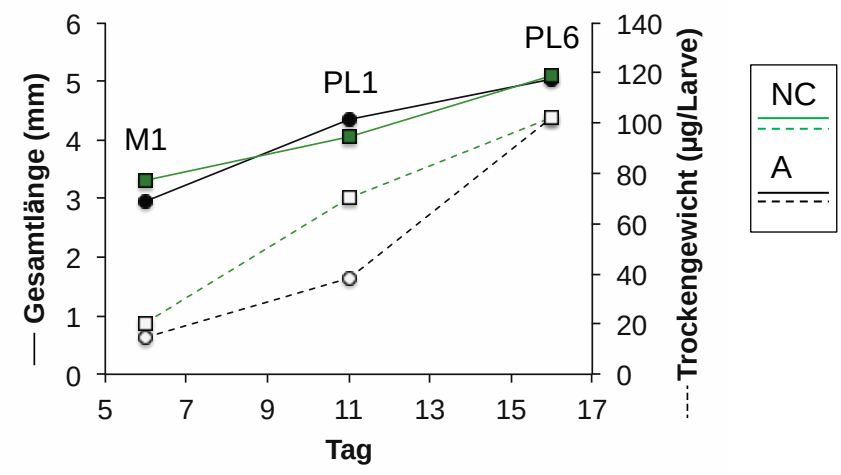

Abb. 21 **Länge und Trockengewicht von** *L. vannamei* Larven nach Fütterung mit Artemien (A) oder angereicherten Nematoden (NC). Dargestellt ist das Wachstum vom Mysis 1 (M1) bis zum Postlarvalstadium 6 (PL6).

Während des Wachstums bis zu Mysis 1 Stadium unterschieden sich die Anteile von DHA, EPA und ARA für die Fütterungsvarianten mit Artemien und die Nematoden innerhalb beider Versuchreihen (3-4) nicht signifikant (Abb. 22-23). Erwartungsgemäß war der DHA-Anteil in den Shrimplarven bei reiner Algenfütterung am höchsten. Signifikante Unterschiede gab es hier zur Fütterung mit Artemien bei der die Shrimps den niedrigsten DHA-Anteil aufwiesen.

Bei fortschreitendem Wachstum der Shrimplarven bis zur PL1 konnten durch die Fütterung von Nematoden signifikant höhere Anteile von DHA, EPA und ARA in den Shrimplarven erzeugt werden als bei Artemienfütterung (Abb. 22-23). Dies galt erstaunlicher Weise auch bei Fütterung von nicht angereicherten Nematoden (N) (Abb. 22). Da die Nematoden allein nicht für den höheren DHA-Anteil verantwortlich sein können, muss davon ausgegangen werden, dass die Shrimplarven zwischen Mysis 1 und PL1 anteilig mehr Algen gefressen haben wenn Nematoden statt Artemien verfüttert wurden oder dass die nicht angereicherten Nematoden Mikroalgen gefressen haben bevor diese dann von den Shrimplarven gefressen wurden. Dies bedarf noch weiterer Untersuchungen. Bei PL6-Larven war der DHA, EPA und ARA-Anteil bei Fütterung mit Nematoden ebenfalls höher als bei Artemienfütterung (Abb. 23).

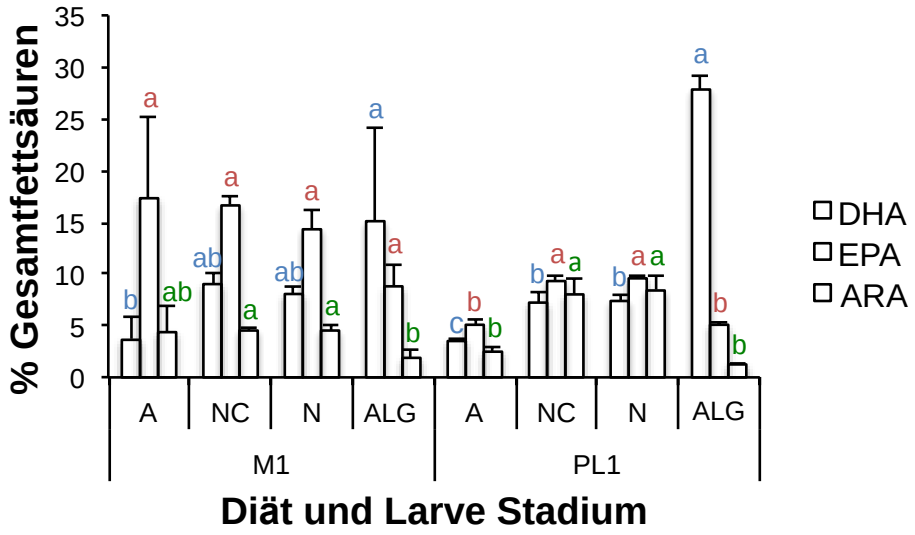

Abb. 22 **Versuch 3:** Relativer Anteil von DHA, EPA und ARA an den Gesamtfettsäuren (Mittel ± SD) in *L. vannamei* Larven nach Fütterung über 6 (M1) und 11 (PL1) Tagen mit unterschiedlichen Diäten bei 30-31°C. Verschiedene Buchstaben über den Datenpunkten zeigen signifikante Unterschiede an ($P < 0,05$).

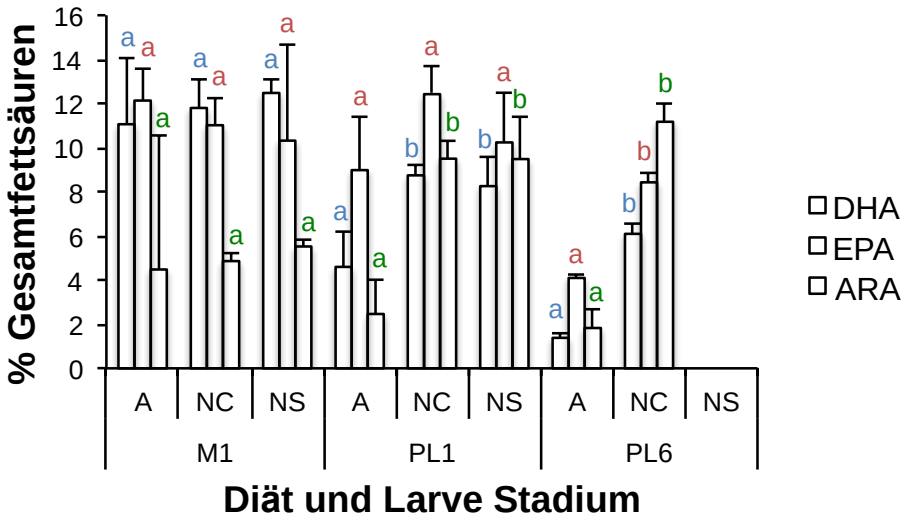

Abb. 23 **Versuch 4:** Relativer Anteil von DHA, EPA und ARA an den Gesamtfettsäuren (Mittel ± SD) in *L. vannamei* Larven nach Fütterung über 6 (M1) und 11 (PL1) Tagen mit unterschiedlichen Diäten bei 30-31°C. Verschiedene Buchstaben über den Datenpunkten zeigen signifikante Unterschiede an ($P < 0,05$).

Im Vergleich zu Wildfängen von *L. vannamei* PL10 Stadien (Montaño and Navarro 1996), war der mit angereicherten Nematoden erzielte DHA-Gehalt in PL6 nur halb so hoch aber der ARA-Gehalt 3-mal so hoch. Hinsichtlich EPA gab es hingegen kaum Unterschiede.

Arachidonsäure (ARA) ist in mehrfacher Hinsicht essentiell für Penaeidea-Garnelen (D'Souza and Loneragan 1999, González-Félix et al. 2009, Bell and Sargent 2003). Unter anderem induziert ARA eine Immunreaktionen bei *Vibrio* Infektionen (Nonwachai et al. 2010) und die Cortisolsynthese und verbessert die physiologische Reaktion auf abrupte Veränderungen der Salinität (Van Anholt et al. 2004).

2.4.2.3 Schlußfolgerungen aus den Fütterungsversuch

Die Fütterungsversuche haben gezeigt, dass Nematoden, bei ausreichender Dichte, ein besseres Zusatzfutter darstellen als Artemia-Nauplien (Abb. 19). Während der Entwicklung von *L. vannamei* vom Z1 bis M1 Stadium (Abb. 18) wurde durch die Gabe von Nematoden höhere Wachstumsraten erzielt. Vor allem aber war der Anteil an Larven erheblich größer, der bis zum 6. Tage das Mysis 1 Stadium erreicht hatte. Beides kann wahrscheinlich unter anderem darauf zurückgeführt werden, dass die Nematoden aufgrund ihrer geringeren Größe schon 2 Tage vor den Artemien an die Shrimplarven verfüttert werden konnten. Dabei spielte es noch keine Rolle, ob die Nematoden angereichert wurden oder nicht. Die kleinen Shrimplarven sind möglicherweise in der Lage ihren Nahrungsbedarf zu größeren Teilen auch über die gefütterten Algen zu decken, wenn Nematoden anstelle von Artemien zugefüttert

werden oder die Mikroalgen werden von den Nematoden gefressen bevor diese dann von den Shrimplarven gefressen werden.

Bei der Entwicklung vom Mysis 1 Stadium zum PL1 Stadium waren angereicherte Nematoden besser geeignet als Futterzusatz als nicht-angereicherte (Abb. 20, Versuch 3). Allerdings erzielte die Fütterung mit Artemien nun geringfügig bessere Ergebnisse hinsichtlich der Entwicklung von *L. vannamei.* Die Anteile von DHA, EPA und ARA in den Shrimps waren aber bei Nematodenfütterung deutlich besser. Bei der Weiterentwicklung vom PL1 zum PL6 Stadium konnten keine Unterschiede zwischen Artemien- und Nematodenfütterung festgestellt werden, aber auch hier war das Fettsäuremuster in den Shrimplarven besser bei Fütterung von Nematoden (Abb 21, 23). Bei der Aquakultur von Shrimps werden ab dem PL1 Stadium diverse Nahrungsadditive gefüttert. Die Überlebensrate von PL1 zu PL6 beträgt dann über 50%, während in unseren Versuchen im Mittel nur 39 % erzielt wurden. Unsere Versuche zeigen aber, dass sich Nematoden als Nahrungsadditiv eignen und dass sie in der Lage sind, das Fettsäuremuster in den Shrimplarven deutlich zu verbessern. Dies könnte die Überlebensrate und die Stresstoleranz der Shrimplarven in Aquakultur erheblich verbessern. Der ARA-Anteil bei den Fettsäuren konnte z. B. annähernd verdreifacht werden. ARA ist involviert in die Stresstoleranz und Osmoregulation. Beides ist relevant während der kritischen Entwicklungsphase von PL5 zu PL6, während der sich die Exkretionsorgane und Kiemen funktionell vollständig ausdifferenzieren (AQUACOP et al. 1991). Nach erfolgreichem Abschluss des PL6 Stadiums ist die Mortalität während weiterer Häutungen üblicherweise gering.

Es ist weiterhin zu beachten, dass der Einsatz von angereicherten Nematoden für den Shrimpzüchter sehr viel einfacher ist als die Verwendung von Artemien. Für die Nematoden werden nur 30 Minuten zur Rehydrierung in Meerwasser benötigt bevor sie verfüttert werden können. Bei Artemien müssen im Gegensatz dazu zunächst die Zysten mit Hypochlorit behandelt und dann über 24 Stunden in Meerwasser bei Licht und starker Belüftung inkubiert werden bis die Nauplien schlüpfen.

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Anhang

Publikationen, die im Rahmen des Projekts entstanden sind.

1. Ayub, F., O. Strauch, L. Seychelles and R. U. Ehlers (2013). Influence of temperature on life history traits of the free-living, bacterial-feeding nematode *Panagrolaimus* sp. strain NFS 24-5. Nematology 14: 939-946.

2. Ayub, F., L. Seychelles, O. Strauch, L. Seychelles and R. U. Ehlers (2014). Influence of cell density of Escherichia coli and the dinoflagellate Crypthecodinium cohnii on life history traits of the nematode Panagrolaimus sp. strain NFS 24-5, a potential larval food for marine aquaculture. Nematology 16: 419-426.

3. Ayub, F., L. Seychelles, O. Strauch, M. Wittke and R. U. Ehlers (2013). Monoxenic liquid culture with *Escherichia coli* of the free-living nematode *Panagrolaimus* sp. (strain NFS 24-5), a potential live food candidate for marine fish and shrimp larvae. Applied Microbiology and Biotechnology 97: 8049-8055.

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Influence of temperature on life history traits of the free-living, bacterial-feeding nematode *Panagrolaimus* sp. strain NFS-24

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Summary – Life cycle analysis data of the free-living, bacterial-feeding *Panagrolaimus* sp. strain NFS 24-5 were assessed at different temperatures using a hanging drop method with single male and female individuals and a food density of 3×10^9 *Escherichia coli* cells ml^{-1}. Lifespan at the moment when the first egg was laid was 5.7 days at 21°C and 4 days at 25, 27 and 29°C. The intrinsic rate of natural increase (r_m) was 0.53 at 21°C, 0.81 at 25°C, 0.93 at 27°C and 0.81 at 29°C, corresponding to population doubling times (PDT $= \ln 2/r_m$) of 1.3, 0.9, 0.7 and 0.9 days, respectively. Over 200 offspring per female were produced at 27°C. All other temperatures yielded fewer offspring. When females were kept without males, the life span was 49 days, whereas the last reproductive female (hanging drop with male individual) died after 16.5 days. These data will contribute to the interpretation of nematode population dynamics recorded in liquid culture.

Keywords – aquaculture, fecundity, generation time, intrinsic rate of natural increase, life history trait analysis, lifespan, offspring production, population doubling time.

As catches of marine fish have decreased during the past decades, aquaculture production is gaining importance. Future production of crustaceans and fish species depends on increasing demands for high quality food. During the early stages of development, marine crustacean and fish larvae rely on live food. Feeding of larval stages of the brine shrimp *Artemia* is standard feed for crustacean larvae all over the world. However, natural resources are limited (Focken *et al.*, 2006). A possible alternative to *Artemia* are free-living, bacterial-feeding nematodes (Rhabditida). Biedenbach *et al.* (1989) used *Panagrellus redivivus* L. with algae as a larval diet for the white shrimp *Litopenaeus vannamei* Boone and found significantly better results compared to the typical *Artemia* diet. However, this nematode cannot be desiccated. Therefore, Honnens & Ehlers (2013) proposed to use the free-living nematode *Panagrolaimus* sp. (strain NFS 24-5) as a potential food organism as it can easily survive desiccation (Honnens *et al.*, 2013) and thus facilitate the development of a product formulation that is stable during storage and transport. First results on *in vitro* propagation of strain NFS 24-5 are available (Honnens & Ehlers, 2013), but precise knowledge on the biology of this nematode is scarce.

A cost-efficient production process demands maximum yields and short process time (Ehlers, 2001; Hirao & Ehlers, 2009). Optimum temperature is an important factor for improving a mass production process. Knowledge on life history traits and growth parameters of a nematode can possibly improve the mass production process. Life cycle analysis data provides detailed information to understand the biological approach of a nematode species, *i.e.*, the nematode's age at sexual maturity, lifespan, net reproductive rate, total fertility rate, generation time, intrinsic rate of natural increase, population doubling time and somatic growth rate. This study analysed the life cycle of the free-living, bacterial-feeding *Panagrolaimus* sp. strain NFS 24-5 at different temperatures by a recently developed 'hanging drop' method (Muschiol & Traunspurger, 2007) and determined the optimum culture temperature for maximum juvenile production.

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Materials and methods

Establishing the monoxenic culture

Strain NFS 24-5 of *Panagrolaimus* sp. was used for this study (Honnens & Ehlers, 2013). *Escherichia coli* (strain K12), used as food for *Panagrolaimus* sp., was propagated at 25°C in 100 ml Erlenmeyer flasks containing 25 ml LB medium (10 g tryptone; 5 g yeast extract; 5 g NaCl; 1 l distilled water) and incubated for 24 h on a rotary shaker at 200 rpm. The monoxenic culture of *Panagrolaimus* sp. was established following a modified surface sterilisation method for nematode eggs originally described for entomopathogenic nematodes by Lunau *et al.* (1993). Polyxenic cultures of the nematodes were grown on NGM (nematode growth medium) containing 3 g NaCl; 17 g agar; 2.5 g soy peptone on 975 ml distilled water in agar plates. After 4 days, the culture plates were flooded with Ringer's solution (9 g NaCl; 0.42 g KCl; 0.37 g $CaCl_2 \cdot 2H_2O$; 0.2 g $NaHCO_3$; 1 l distilled water). Eggs, juveniles and adults were sucked from the plates and passed through a 41 μm sieve. Eggs and young first-stage juveniles (J1) passed through the sieve. By sucking off the J1 using a capillary tube, eggs were separated from the remaining J1. The eggs were sterilised for 4 min using a sterilisation solution (1 ml 12% NaOCl; 1 ml 4 M NaOH; 10 ml distilled water) and subsequently washed twice with sterile yeast-salt (YS) medium (5 g NaCl, 5 g yeast extract, 0.5 g NH_4PO_4, 0.5 g K_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 l distilled water). The eggs were kept in sterile YS for 72 h. During this time, J1 hatched. When the medium stayed clear, an indication for the absence of any contaminating microorganisms, the J1 were transferred to 24-h-old *E. coli* cultures on NGM medium.

Preparation of hanging drops

The hanging drop method was adopted from Muschiol & Traunspurger (2007); *E. coli* cells in exponential growth phase were obtained by centrifugation at 1100 *g* of 24 h cultures in LB medium. The supernatant was discarded and the cells washed once with salt solution (3.1 g NaCl, 2.4 g KCl, 1 l distilled water). The washed *E. coli* pellets were suspended in Nematode Gelrite Growth Medium (NGG) containing 2.5 g casein peptone; 3 g NaCl; 2.5 g gelrite; 995 ml distilled water (Muschiol & Traunspurger, 2007). Cell density was assessed by counting aliquots in a Thoma chamber (0.01 mm depth, Poly Optic). Final bacterial density was adjusted to 3×10^9 cells ml^{-1} by adding NGG. The NGG-*E. coli* mix was stored for a maximum of 4 days at 4°C. Multiwell plates with 12 wells of 2.2 cm diam. (Greiner bio-one, Cellstar®) were used for the life cycle studies. The bottom of each well was filled with 100 μg pieces of dry cellulose tissue paper and soaked with 500 μl tap water in order to avoid desiccation of the hanging drops. Drops of 10 μl of the NGG bacterial mix were placed on the centre of the inner side of the lid over each well. Plates were sealed with Parafilm to reduce water evaporation. To these drops single nematodes or couples of male and females were transferred with an eyelash mounted at the tip of a Pasteur pipette. In all experiments, nematodes were transferred to fresh drops of the bacterial mix daily in order to make available enough food at any time of development.

Determination of reproductive strategy and female lifespan

In order to determine whether the investigated species reproduces by parthenogenesis or automixis, 15 females were kept singly at 25°C in hanging drops. Every day after nematode transfer to fresh hanging drops the drops were observed under a dissecting microscope (Zeiss Stemi SV11) at 40× magnification for presence of offspring. The experiment was continued until the last nematode died. The lethal time when 50% of the females had died was estimated by using the Gompertz curve (Winsor, 1932).

Growth measurement

A total of 20 single J1 were investigated for somatic growth, out of which 11 became males and 9 became females. The experiment was performed at 27°C. Measurement of nematode length was done at room temperature (approximately 20°C).

Offspring production and lifespan at different temperatures

Mature female and male nematodes were randomly selected from NGG plates. Twenty-four pairs were kept in a hanging drop. After 24 h, parental pairs were removed from the drops and only the eggs laid within 24 h were left in the drops. The juveniles hatched after another 24 h (day 1) and without transfer to fresh medium developed into J4 within 72 h (day 3). Fourth-stage juvenile females from this developmentally synchronised population were used for the assessment of the life cycle parameters. Single female individuals were transferred to NGG drops

and single mature males with visible spicules collected from NGG plates were added to each female. One male and one female were kept in each hanging drop containing a food density of 3×10^9 *E. coli* ml^{-1}. Every day, each pair was transferred to a fresh drop for 24 h and the plates containing old drops were kept for another 24 h until juveniles hatched. All the plates were kept in an airtight box after sealing with Parafilm to avoid desiccation. Observation of single females was terminated only when a female had died. If a male died then a new male was introduced again from NGG stock cultures. The experiment was carried out until the last female had died.

Nematode hanging drops were kept at four different temperatures (21, 25, 27 and 29°C). Nematode stock cultures were always acclimatised for 3 weeks prior to switching to a new temperature. The minimum number of couples observed was 25 and the experiment was repeated twice.

Sample fixing

Since it was not possible to count the juveniles of all investigated nematode pairs at the same time, all the samples were fixed for future counting. After hatching of juveniles, the lids containing hanging drops were kept on a heater (Retbasic, Ika Labortechnik) at about 50°C for 10 min to kill all juveniles. Ten μl aqueous Rose Bengal solution (300 μg ml^{-1}; Sigma-Aldrich) was added to each drop to ease counting and subsequently each drop was covered with a 18 mm round cover slip (Thermo Scientific). All multiwell plates were sealed again with Parafilm and all the counting was performed within 7 days after fixation under a dissecting microscope at 20× magnifications. An underlying grid facilitated counting.

Calculation of life cycle parameters and statistical analysis

The intrinsic rate of natural increase (r_m), population doubling time (PDT), total fertility rate (TFR), net reproductive rate (R_0), alternative measures of generation time (T_0, T_1, T), age at first egg deposition (T_{min}) and mean time at which 50% of females died (LT_{50}) were calculated. The intrinsic rate of natural increase (r_m) was calculated using the fundamental equation of population dynamics according to the Euler equation. This equation is also referred to as the Lotka equation (Vranken & Heip, 1983):

$$\sum_{x=0}^{d} e^{-r} m^x l_x m_x = 1$$

where r_m = intrinsic rate of natural increase, x = time (days), l_x = age-specific survival probability, m_x = age-specific fecundity.

A Microsoft Visual basic (6.0) macro provided by Daniel Muschiol, Department of Animal Ecology, University of Bielefeld (Bielefeld, Germany), was used for r_m iteration. Population doubling time (PDT) was calculated according to the formula: $PDT = \ln 2 / r_m$. The total fertility rate, net reproductive rate and generation time were calculated according to the life table constructed with l_x and m_x (data not shown). Total fertility rate (TFR) is the total number of juveniles that would be produced by the females if they were able to survive until the end of their reproductive period: $TFR = \sum m_x$.

Net reproductive rate (R_0) depends on the age-specific survival probability and fecundity, which is defined as the average number of juveniles that a female in a population produces during its life time: $R_0 = \sum l_x m_x$.

Alternative generation time was measured using the following equations (as described by Vranken & Heip, 1983):

1) the mean generation time (T_1) refers to that period of time necessary for a population growing at a constant rate r_m to increase by the factor R_0: $T_1 = \ln R_0 / r_m$;
2) the mean age of mothers in a cohort at birth of female offspring (T_0) (also referred to as T_c, the cohort generation time): $T_0 / T_c = (1/R_0) \sum x l_x m_x$;
3) the age of the mother of an average newborn in an exponentially growing population (T): $T = \sum x e^{-r} m^x \times l_x m_x$.

The LT_{50} value was estimated by using Gompertz model (Winsor, 1932). Effects of temperature on life cycle parameters were analysed using analysis of variance (ANOVA) and the Tukey's honestly significant differences (HSD) test for unequal n.

Results

Reproduction biology and lifespan

None of the 15 singly kept females produced any offspring. Therefore, automictic and parthenogenetic reproduction can be excluded. Compared with reproductive females, the non-reproductive individuals had a prolonged lifespan (Fig. 1). The first non-reproductive female died at day 14, whereas the first reproductive female had already died at day 6 (Fig. 1). The last non-reproductive female died at day 49. Compared with non-reproductive

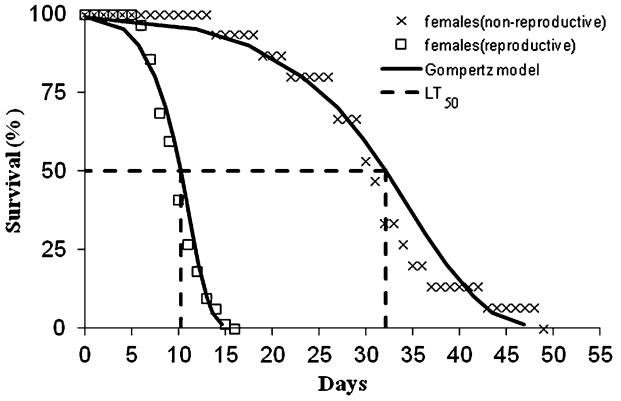

Fig. 1. Survival (in %) of *Panagrolaimus* sp. (strain NFS 24-5) at 25°C assessed daily of non-reproductive ($n = 15$) and reproductive females ($n = 91$). Gompertz model and lethal time at which 50% survival is recorded (LT_{50}). Each square or cross stands for % survival for that corresponding day.

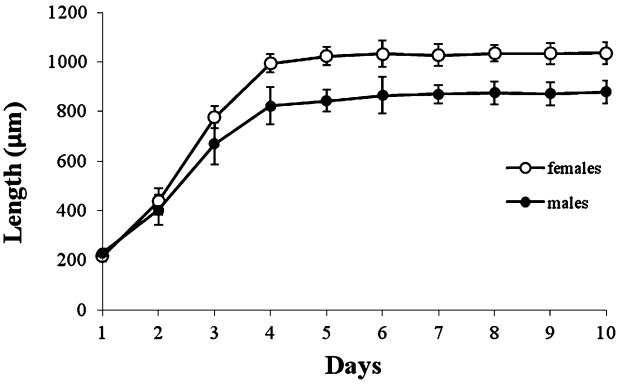

Fig. 2. Development of total length of male ($n = 11$) and female ($n = 9$) *Panagrolaimus* sp. (strain NFS 24-5) over time (days) measured from cultures grown at 27°C. Error bars indicate standard deviation of the mean.

females, the reproductive females had a higher mortality rate, which is also indicated by the LT_{50} values (lethal time for 50% of the population), which was 10.1 days for reproductive females and 32.1 days for non-reproductive females.

Somatic growth

The somatic growth is shown in Figure 2. At the beginning of the measurement (after 24 h), the lengths of all juveniles were almost the same (female 217 ± 23.2, male 230 ± 17.3 μm). Adult females (1035 ± 43.8 μm) were longer than adult males (879 ± 46.8 μm). Sex of the nematode juvenile stages was distinguishable after 3 days, though they needed 4 days to reach the adult stage. Juvenile growth was almost linear until day 4 when they became adults. Both, males and females reached adulthood within the same developmental time period.

Population growth parameters

Data on the life cycle parameters at different temperatures are presented in Table 1, and the cumulative offspring production in Figure 3. At 21°C the ontogenesis and lifespan of the nematodes were significantly longer ($P > 0.05$), as can be seen from the generation time parameters (T_{min}, T_0, T_1, T) and the LT_{50} (Table 1). The highest LT_{50} of 15.6 days was obtained at 21°C, but at 27°C the LT_{50} was higher than at 25 and 29°C (not significant; $P < 0.05$). In most cases (except for TFR and R_0) life cycle parameters at 21°C were significantly different ($P > 0.05$) from those recorded at higher temperatures, whereas no significant differences were obtained among the temperatures 25, 27 and 29°C. The maximum total fertility rate TFR of 259 offspring per female was calculated for 27°C. The maximum net reproductive rate R_0 of 213.6 offspring per female was calculated for the same temperature and was significantly higher ($P > 0.05$) than at 21, 25 and 29°C. It corresponds well with the cumulative mean number of offspring presented in Figure 3. In general, 27°C seems to be close to the optimal growth temperature of this nematode since parameters regarding the generation time (T_0, T_1, T) were found to be lower than at the other tested temperatures, whereas offspring production represented by TFR and R_0 was higher, although not always statistically significant. Also, the intrinsic rate of natural increase, r_m, which combines generation time and offspring production, was the highest at 27°C and the population doubling time was the lowest at 27°C.

Discussion

Temperature plays an important role in the reproductive strategy of nematodes (Brown *et al.*, 2004). At a much lower or higher temperature, fecundity and reproduction can cease due to infertility or sterility of the individuals (Greet, 1978). Temperature also has a profound effect on minimum generation time (T_{min}). The value can range from 1.5 days at higher temperature to more than 100 days at lower temperature (Heip *et al.*, 1985). For *Panagrolaimus* (strain NFS 24-5) a value of 4.0 days was recorded for T_{min} (from egg to egg) at 25, 27 and 29°C and 5.7 days at 21°C, which is the shortest T_{min} reported for a *Panagrolaimus* sp. (Table 2).

Table 1. Life cycle parameters of *Panagrolaimus* sp. (strain NFS 24-5) assessed at different temperatures.

	Temperature (°C)				$F_{3,8}$
	21	25	27	29	
n	75	91	107	137	
T_{min} (days)	5.7 ± 0.6^{a}	4.0 ± 0.0^{b}	4.0 ± 0.0^{b}	4.0 ± 0.0^{b}	25.00
LT_{50} (days)	15.6 ± 1.7^{a}	10.1 ± 1.1^{b}	11.1 ± 1.0^{b}	9.2 ± 0.4^{b}	19.15
TFR	157.1 ± 19.6^{ab}	218.5 ± 32.6^{ac}	259.0 ± 26.0^{c}	132.7 ± 29.2^{b}	13.33
R_0	119.0 ± 10.4^{ab}	156.3 ± 21.0^{a}	213.6 ± 4.8^{c}	97.5 ± 31.5^{b}	19.85
r_m (day^{-1})	0.53 ± 0.0^{a}	0.81 ± 0.0^{b}	0.93 ± 0.0^{b}	0.81 ± 0.1^{b}	23.71
T_0 (days)	10.3 ± 0.7^{a}	7.1 ± 0.4^{b}	6.8 ± 0.1^{b}	6.3 ± 0.4^{b}	49.20
T_1 (days)	9.1 ± 0.6^{a}	6.2 ± 0.2^{b}	6.1 ± 0.3^{b}	5.6 ± 0.3^{b}	45.92
T (days)	8.2 ± 0.5^{a}	5.7 ± 0.2^{b}	5.3 ± 0.1^{b}	5.2 ± 0.3^{b}	63.36
PDT (days)	1.3 ± 0.1^{a}	0.9 ± 0.05^{b}	0.7 ± 0.02^{b}	0.9 ± 0.11^{b}	27.95

Values represent mean from three replicates ± SD. Different superscript letters indicate significant differences between values within one line (*post hoc* Tukey's HSD test, $P \leqslant 0.001$). n = number of females observed; T_{min} = age at first egg deposition; lifespan (LT_{50}) = mean time at which 50% of females died; TFR = total fertility rate; R_0 = net reproductive rate; T_0 = mean age at reproduction of a cohort of females (also referred to as T_c, the cohort generation time); T_1 = period of time necessary for a population growing at a constant rate r_m to increase by the factor R_0; T = mean age of the mothers of a set of newborn individuals in a population with a stable age distribution; r_m = intrinsic rate of natural increase; PDT = population doubling time.

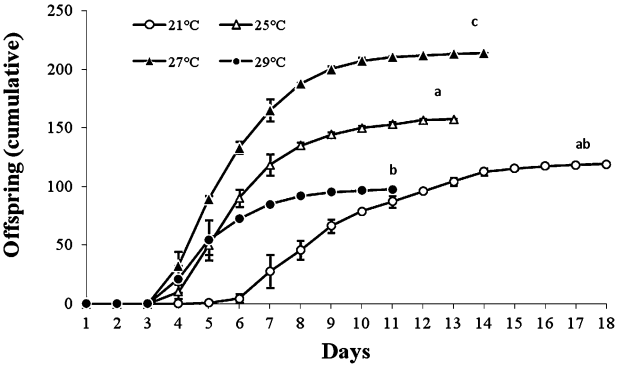

Fig. 3. Cumulative mean offspring production per female nematode assessed in hanging drops with 3×10^9 cells ml^{-1} of *Escherichia coli* at different culture temperatures of 21°C ($n = 75$), 25°C ($n = 91$), 27°C ($n = 107$) and 29°C ($n = 137$). Error bars indicate standard deviation of the mean. Different letters indicate statistical differences between cumulative offspring numbers assessed on the last day of egg laying of the females at different temperature (post hoc Tukey's HSD test, $P \leqslant 0.05$).

Table 2. Age of female nematode at first egg laying (T_{min}) measured at different temperature for different *Panagrolaimus* species.

Species	Temp. (°C)	T_{min} (days)	Reference
Panagrolaimus sp.[1]	25, 27, 29	4	Present study
Panagrolaimus sp.[1]	21	5.7	Present study
P. davidi	25	7	Brown *et al.* (2004)
P. detritophagus	20	7	Sohlenius (1988)
P. superbus	20	8	Sohlenius (1988)
P. australis	20	8.8	Yeates (1970)
P. davidi	20	9	Brown *et al.* (2004)
Panagrolaimus sp.[2]	20	9.5	Muschiol & Traunspurger (2007)

[1] Isolated from soil in Hoheneichen, Germany and used in the present study.

[2] Isolated from chemoautotrophic microbial mats at the Movile Cave, Dobrogea, Romania.

Strain NFS 24-5 had highest TFR (259.0) and R_0 (213.6) values at 27°C, which decreased to 132.7 and 97.5, respectively, at 29°C. Our values of TFR (157.1) and R_0 (119.0) at 21°C were higher than the reported values of TFR (77.0) and R_0 (64.0) by Muschiol & Traunspurger (2007) at 20°C for their *Panagrolaimus* sp., but R_0 was lower compare to *Panagrolaimus australis* (195.6) cultured at 20°C (Yeates, 1970). On the other hand, our R_0 value of 156.3 at 25°C was higher than the R_0 value of 142.0 for *P. davidi* (Brown *et al.*, 2004) at the same temperature.

From these results on the life cycle parameters (*i.e.*, high fecundity, small body size, early maturity onset, short generation time), it can be concluded that *Panagrolaimus* sp. (strain NFS 24-5) possesses classical *r*-selected features; *r*-selected features for *Panagrolaimus* nematodes

have also been observed by Brown *et al.* (2004) for the Antarctic nematode *P. davidi* and by Schroeder *et al.* (2010) for a Movile Cave *Panagrolaimus* sp. Free-living *Panagrolaimus* spp. are available in soils in most ecosystems ranging from Antarctic, *P. davidi* (Brown *et al.*, 2004), to coastal sand dunes, *P. australis* (Yeates, 1970).

The results indicate that 27°C is the most favourable temperature for *Panagrolaimus* sp. in terms of maximum juvenile production. Brown *et al.* (2004) reported highest numbers of juvenile production for *P. davidi* between 20-25°C and Greet (1978) between 15-20°C for *P. rigidus*. A higher number of eggs per female at higher temperature was also observed by Heip *et al.* (1985). They mentioned that effects of temperature were species-dependent and they showed that *M. microphthalma* is very temperature-dependent, *Diplolaimelloides bruciei* intermediate and *Oncholaimus oxyuris* is only slightly affected. Hirao & Ehlers (2009) observed highest fecundity for a commercially cultured rhabditid nematode, *Steinernema carpocapsae*, at 27°C, although maximum yields were obtained at 25°C. Lowest juvenile production was obtained at 29°C which was probably too high for strain NFS 24-5 as well, as high temperature can affect male sperm fertility (Prasad *et al.*, 2011).

The highest value of an intrinsic rate of natural increase (r_m) was obtained at 27°C (0.925), although the value was not significantly different from the values obtained at 25°C and 29°C. For an Antarctic species, *P. davidi*, Brown *et al.* (2004) reported a r_m value of 0.48 at 25°C, which is lower compared to our value 0.81 at the same temperature. The slowest population doubling time (PDT) of 1.3 days was obtained at the lowest temperature (21°C), which was still faster compare to *Panagrolaimus* sp. isolated from a cave (2.24 days) and cultivated at 20°C (Muschiol & Traunspurger, 2007). The highest PDT value of 0.7 days was also observed at 27°C.

Reproductive strategy and behaviour of a nematode species are two important factors when developing liquid culture production. In some cases, as for *Heterorhabditis* spp., amphimictic adults cannot copulate in liquid culture (Strauch *et al.*, 1994). The results with single females in a hanging drop confirmed that the nematode strain NFS 24-5 is reproducing amphimictically. Parthenogenesis, as reported for an Antarctic nematode *P. davidi* (Goldstein & Wharton, 1996; Brown *et al.*, 2004), can be excluded as well.

Non-reproductive females had a much longer life span (49 days) compared with the reproductive ones (16 days) at 25°C. Woombs & Laybourn-Parry (1984) showed the same results with *Diplogasteritus nudicapitatus*, *Paroigolaimella bernensis* and *Rhabditis curvicaudata*. Reported values of total life span for reproductive *P. superbus* and for *P. detritophagus* at 20°C are 16-17 days and 16 days, respectively (Sohlenius, 1988). Both values are similar to the results reported in this study. With higher temperature the lifespan is reducing. Adulthood was reached after 4 days. Egg laying of *Panagrolaimus* (strain NFS 24-5) started as soon as sexual maturity was reached, which is supported by the data of Brown *et al.* (2004) for *P. davidi*. Increase in length of *Panagrolaimus* (strain NFS 25-5) seems to be a continuous process and juvenile stages could not be distinguished as reported before from measurements of juveniles originating from liquid culture (Honnens & Ehlers, 2013). Continuous growth curves for nematodes are reported by many authors (*e.g.*, Woombs & Laybourn-Parry, 1984; Muschiol & Traunspurger, 2007). However, the 24 h time resolution might be too long to observe minor fluctuations in the growth rate (Muschiol & Traunspurger, 2007) compared to 35 min intervals reported by Knight *et al.* (2002) for *Caenorhabditis elegans*. Observations every 24 h might hide the details as discontinuities in the growth curves of *C. elegans* and *Panagrellus redivivus* during molting was reported by Wilson (1976). The onset of reproduction coincided with the end of the linear growth phase as reported by Muschiol & Traunspurger (2007) for a *Panagrolaimus* sp. Average length of the females until 10 days was 1000 μm, which was smaller than the lengths of another *Panagrolaimus* sp. (1688 μm) reported by Muschiol & Traunspurger (2007).

Our main goal is to introduce this nematode in aquaculture as live food for commercially important marine shrimps. For that purpose they need to be produced on a large scale in liquid culture condition in bioreactors. Data from our study can provide basic information for improvement of large-scale culture. Studying *Panagrolaimus* sp. by means of the 'hanging drop' method is especially suitable for life cycle experiments, which facilitates the observation of single females and provides precise data about juvenile production at defined conditions. From the results, we can conclude that at optimum conditions (*i.e.*, at 27°C with a food density of 3×10^9 cells ml^{-1}) *Panagrolaimus* sp. (strain NFS 24-5) can produce more than 200 juveniles per female on average in its lifetime. Comparison of the data on LHT with results obtained by Honnens & Ehlers (2013) in liquid culture points to large discrepancies and might be useful to overcome problems with liquid culture mass production. LHT results indicate generation time of approximately 6 days at 25°C. In liquid

culture, major reproduction started only 9 days after inoculation. The reasons for the prolonged lag phase observed in liquid culture need to be investigated and a closer look at the population development might help us to understand why nematode density hardly increases over approximately two generations.

The possible reproduction in liquid culture inoculated with 4000 individuals (Honnens & Ehlers, 2013) with the net reproduction rate of 150 juveniles per female and a sex ratio of 1:1 would result in a maximum yield of 3×10^5 nematodes ml^{-1} within approximately 1 week. Instead, the yields of Honnens & Ehlers (2013) varied between 45 and 238 $\times$ 10^3 ml^{-1} for a culture period of 13-15 days. Another reason for differences, which cannot of course be excluded as a reason for deviation, is the food organism, which was *E. coli* for this study and *S. cerevisiae* in the study of Honnens & Ehlers (2013). In conclusion, the LHT analysis provided valuable information on optimal growth temperature and possible maximum yields in liquid culture mass production.

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Influence of cell density of *Escherichia coli* and the dinoflagellate *Crypthecodinium cohnii* on life history traits of the nematode *Panagrolaimus* sp. strain NFS 24-5, a potential larval food for marine aquaculture

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Summary – The nematode *Panagrolaimus* sp. NFS 24-5 has potential for use as living food for larval shrimps and fish in marine aquaculture. The nematodes are usually produced on bacterial or yeast cells. Nematodes cannot synthesise the long chain fatty acid docosahexaenoic acid (DHA) which is essential for feeding marine aquaculture organisms. The eukaryotic, heterotrophic dinoflagellate *Crypthecodinium cohnii* consists of approximately 20% DHA. To culture the nematodes and simultaneously enrich them with DHA, single adult male and female individuals were cultured in hanging drops with variable cell density of *C. cohnii*. Life history traits, such as net reproductive rate (R_0), population doubling time (PDT) and intrinsic rate of natural increase (r_m), were assessed and compared with data obtained from cultures on *Escherichia coli*. A maximum R_0 was recorded at a cell density of 4×10^6 *C. cohnii* cells ml^{-1}, corresponding to 2478.82 μg dry mass ml^{-1}. The same was achieved with 7× lower biomass of *E. coli* at a cell density of 3×10^9 cells ml^{-1}, corresponding to 335.63 μg dry mass ml^{-1}. The results exclude the use of the dinoflagellate culture from application in mass production of the nematode for aquaculture food and limit the use to post-harvest enrichment of the nematodes with essential fatty acids. At a density of 3×10^9 *E. coli* cells ml^{-1} the PDT was lowest and the r_m was highest, indicating that this cell density might be closest to optimum conditions for nematode reproduction. Exceeding this cell density yielded fewer offspring within a longer time period. Implications for mass production in monoxenic liquid cultures are discussed.

Keywords – food density, intrinsic rate of natural increase, lifespan, net reproductive rate.

With the growing demand for crustacean and fish species for human nutrition, their production in aquaculture is steadily increasing. The major bottleneck in aquaculture of marine fish and shrimps is the feeding of the larval stages. They require living food organisms and the production of these significantly contributes to the overall costs (Guillaume & Métailler, 2001). The most common prey is the brine shrimp *Artemia salina*. It is harvested from hypersaline lakes. Their quality can vary depending on environmental conditions and the shrimp can carry contaminants and lethal pathogens (Diggles *et al.*, 2000; Lavens & Sorgeloos, 2000; Villamil *et al.*, 2003). Therefore, nematodes have been considered as an alternative food for marine fish and shrimp larvae (Fisher & Fletcher, 1995; Focken *et al.*, 2006). As some nematode species can be mass produced on an industrial scale in monoxenic liquid culture (Ehlers, 2001) the transmission of pathogens can be excluded. Feeding trials reported by Biedenbach *et al.* (1989) and Focken *et al.* (2006), using *Panagrellus redivivus* as life feed for the shrimp *Litopenaeus vannamei*, provided encouraging results. This nematode species, however, cannot be desiccated like *Artemia*. Therefore, the free-living bacterial-feeding nematode *Panagrolaimus* sp. strain NFS 24-5, which is highly tolerant to desiccation (Honnens *et al.*, 2013a), was selected for further development for use as larval prey (Honnens & Ehlers, 2013a). Yields obtained during *in vitro* cultivation in flasks (Honnens & Ehlers,

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2013a) and bioreactors (Honnens & Ehlers, 2013b) varied for reasons that have not yet been identified. To understand the reproductive biology better, life history traits of *Panagrolaimus* sp. strain NFS 24-5 were assessed at variable temperatures (Ayub *et al.*, 2013a). The objective of the present investigation was to assess the influence of variable food density on life history traits of this nematode.

Nematode growth depends on the availability of food organisms of appropriate size; mainly bacteria but also yeast, algae or multicellular organisms are known food resources (Fürst von Lieven, 2003; Moens *et al.*, 2006). Different levels of food supply have a significant impact on population growth (Venette & Ferris, 1998; Schroeder *et al.*, 2010) and on a cost-efficient production process, which demands maximum yields within a short process time (Hirao & Ehlers, 2009). However, not all types of bacteria are suitable as food for nematodes (Andrew & Nicholas, 1976). Laboratory nematode cultures are usually based on *Escherichia coli*. It is known to support growth of free-living nematodes such as *Caenorhabditis elegans*, *Panagrellus redivivus* or *Panagrolaimus* sp. (Cryan *et al.*, 1963; Andrew & Nicholas, 1976; Schiemer, 1982; Ferris *et al.*, 1997; Carta, 2000; Muschiol & Traunspurger, 2007).

Any kind of living food organisms for marine fish or shrimps need to provide fatty acids essential for larval development. One shortcoming of nematodes is that their nutritional composition is not optimal for marine fish and shrimps. Free-living nematodes such as *P. redivivus*, *C. elegans* and *Panagrolaimus* sp. (strain NFS 24-5) synthesise arachidonic acid (ARA, C 20:4n6) and eicosapentaenoic acid (EPA, C 20:5n3), but cannot synthesise docosahexaenoic acid (DHA, C 22:6n3) (Rouse *et al.*, 1992; de Lara *et al.*, 2007; Honnens *et al.*, 2013b). Thus, DHA has to be incorporated into the nematode diet if they are the sole food item for marine fish and shrimp larvae. *Panagrolaimus* sp. was successfully enriched with DHA by feeding nematodes harvested from yeast cultures with the algal extract S.presso® (Inve Aquaculture) (Honnens *et al.*, 2013b). The present study tested feeding of nematodes with the marine dinoflagellate *Crypthecodinium cohnii* for enrichment with DHA. The dry mass of *C. cohnii* consists of approximately 20% DHA. This eucaryotic alga is heterotrophic, needs no light for growth and can therefore be produced in conventional bioreactors (de Swaaf *et al.*, 1999). If used as food source, nematodes would automatically be enriched with DHA. This is of particular interest, as the main live preys (*Artemia* or rotifers) used for larval feeding carry opportunistic and potentially pathogenic bacteria, of which some are opportunistic (Vadstein *et al.*, 2004). In addition, the bacterial density can increase during fatty acid enrichment (Ritar *et al.*, 2004; Seychelles *et al.*, 2013). The present study compared life history traits of the nematode *Panagrolaimus* strain NFS 24-5 culture on *E. coli* or *C. cohnii* at different food density, using a recently developed 'hanging drop' method (Muschiol & Traunspurger, 2007) to observe development and life history traits of single nematodes.

Materials and methods

Establishing monoxenic cultures

Strain NFS 24-5 of *Panagrolaimus* sp. was used for this study (Honnens & Ehlers, 2013a). Pure cultures of *E. coli* (strain K12) and *C. cohnii* (ATCC 30772) were used as food for *Panagrolaimus* sp. *Escherichia coli* was propagated on sterile LB medium (10 g trypton; 5 g yeast extract; 5 g NaCl; 1 l distilled water) for 24 h and *C. cohnii* was cultured for 4 days in a sterile medium containing 2 g yeast extract; 18 g sea salt; 9 g glucose and 1 l distilled water. Both organisms were cultured at 25°C in 100 ml Erlenmeyer flasks containing 25 ml medium on a rotary shaker at 200 rpm.

The monoxenic culture of *Panagrolaimus* sp. was established following a modified surface sterilisation method for nematode eggs originally described for entomopathogenic nematodes by Lunau *et al.* (1993). Polyxenic cultures of the nematodes were grown on nematode growth medium (NGM) containing 3 g NaCl, 17 g agar, 2.5 g soya peptone and 975 ml distilled water on agar plates. After 4 days, the culture plates were flooded with Ringer's solution (9 g NaCl, 0.42 g KCl, 0.37 g $CaCl_2 \cdot 2H_2O$, 0.2 g $NaHCO_3$, 1 l distilled water). Eggs, juveniles and adults were sucked from the plates and passed through a 41 μm sieve. Eggs and young first-stage juvenile (J1) stages passed through the sieve. By sucking off the J1 using a capillary tube, eggs were separated from the remaining juveniles. The eggs were sterilised for 4 min using a sterilisation solution (1 ml 12% NaOCl, 1 ml 4 M NaOH, 10 ml distilled water) and subsequently washed twice with sterile yeast salt medium (YS) (5 g NaCl, 5 g yeast extract, 0.5 g NH_4PO_4, 0.5 g K_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 l distilled water). The eggs were then kept in sterile YS for 72 h. During this time J1 hatched. When the medium stayed clear (an indication for the absence of any contaminating microorganisms) and after checking

for bacterial contamination under an inverted microscope (Zeiss Axiovert 25) at 200× magnification, the juveniles were transferred to 24 h old *E. coli* or *C. cohnii* cultures on NGM medium.

Preparation of hanging drops

The hanging drop method was adopted from Muschiol & Traunspurger (2007). Cells of *E. coli* in exponential growth phase were obtained by centrifugation at 1100 *g* of 24 h pure cultures in LB medium. The supernatant was discarded and the cells washed once with sterile salt solution (3.1 g NaCl, 2.4 g KCl, 1 l distilled water). The washed *E. coli* pellets were suspended in sterile nematode growth gelrite (NGG) containing 2.5 g casein peptone; 3 g NaCl, 2.5 g Gelrite, 995 ml water (Muschiol & Traunspurger, 2007). Four-day-old pure *C. cohnii* cultures were sieved under sterile conditions through a 10 μm sieve using a vacuum pump to suck through the sieve the swimming cells of 5-10 μm diam. Cell size was measured under the microscope at 400× magnification by the use of an image analysing system analySIS (Soft Imaging). Cells were centrifuged and re-suspended in sterile NGM and the density of well mixed suspensions was assessed by counting two aliquots in a Thoma chamber (Poly Optic; 0.01 mm depth for *E. coli* and 0.1 mm for *C. cohnii*). Final cell densities were adjusted by adding NGG. The NGG-*E. coli*/*C. cohnii* mixes were stored for a maximum 4 days at 4°C.

Sterile multiwell plates with 12 wells of 2.2 cm diam. (Cellstar®, Greiner Bio-one) were used for the life cycle studies. The bottom of each well was filled with 100 μg pieces of dry cellulose tissue paper and soaked with 500 μl tap water in order to avoid desiccation of the hanging drops. Drops of 10 μl of the NGG bacterial/algal mix were placed on the centre of the inner side of the cover lid over each well. To these drops couples of one male and one female were transferred with an eyelash mounted at the tip of a Pasteur pipette. The cover lids were finally placed on top of the cell wells and plates were sealed with Parafilm to reduce water evaporation. In all experiments nematodes were transferred to fresh drops of the bacterial/algal mix daily in order to make available enough food at any time of development. The application of the NGG bacterial/algal mix and the transfer of males and females were carried out with sterile equipment but not under completely sterile conditions. However, the establishment of microbial contaminations is limited because of the high density of *E. coli* or *C. cohnii*, which quickly take all available food reserves from the substrate. Furthermore, the adult nematodes were transferred daily to new NGG drops with pure *E. coli* or *C. cohnii* cultures.

Offspring production and lifespan at different food densities

Mature female and male nematodes were randomly selected from NGG plates. Twenty-four pairs were each kept in a hanging drop. After 24 h, parental pairs were removed from the drops and only the eggs laid within 24 h were left in the drops. The juveniles hatched after another 24 h and without transfer to fresh medium developed into fourth-stage juveniles (pre-adult) within 72 h. These pre-adult females from the developmentally synchronised population were used for the assessment of the life cycle parameters. Single female individuals were transferred to NGG drops and single mature males with visible spicules collected from NGG plates were added to each female. One male and one female were kept in each hanging drop containing the desired bacterial/algal food density. Every day, each pair was transferred to a fresh drop for 24 h and the plates containing old drops were kept for another 24 h until juveniles hatched. All the plates were kept in an airtight box after sealing with Parafilm to avoid desiccation. Observation of single females was terminated only when a female had died. When a male died then a new male was introduced from NGG stock cultures. The experiment was carried out until the last female had died.

Six different *E. coli* food densities, *i.e.*, 10^6, 10^7, 10^8, 10^9, 3×10^9 and 10^{10} cells ml^{-1} that correspond to biomass values of 0.12, 1.12, 11.19, 111.881, 335.63 and 1118.75 μg ml^{-1}, respectively, and four different *C. cohnii* food densities, *i.e.*, 10^5, 10^6, 2×10^6 and 4×10^6 cells ml^{-1} that correspond to biomass values of 61.971, 619.711, 1239.41 and 2478.82 μg ml^{-1}, respectively, were tested to determine optimum food density. Each food density and each replicate was assessed at different times with different nematode and bacteria batches. Data of life history trait parameters obtained with 335.63 μg ml^{-1} (3×10^9 cells ml^{-1}) of *E. coli* were obtained from the investigation by Ayub *et al.* (2013a).

The minimum number of couples observed was 21 and each experiment was repeated twice. All the experiments were performed at 27°C as the best growth of *Panagrolaimus* sp. strain NFS-24-5 was obtained at this temperature (Ayub *et al.*, 2013a).

Sample fixing

Since it was not possible to count the juveniles of all investigated nematode pairs at the same time, all samples were fixed for future counting. After hatching of juveniles, the lids containing hanging drops were put on a heater (Retbasic, Ika Labortechnik) at about 50°C to kill all juveniles. Ten μl aqueous Rose Bengal solution (300 μg ml^{-1}; Sigma-Aldrich) was added to each drop to ease counting and subsequently each drop was covered with a 18 mm round cover slip (Thermo Scientific). All multiwell plates were sealed again with Parafilm and all the counting was performed within 7 days after fixing under a dissecting microscope at 40× magnification. An underlying grid facilitated counting.

Assessment of bacterial and algal biomass

Known volumes of samples with known cell densities, assessed as mentioned above, of *E. coli* and *C. cohnii* were freeze-dried to assess the dry mass.

Calculation of life cycle parameters and statistical analysis

The mean number of days survived by the females (lifespan), net reproductive rate (R_0), age at maximum rate of juvenile production (T_{rate}), alternative measure of generation time (T), intrinsic rate of natural increase (r_{m}) and population doubling time (PDT) were calculated; r_{m} was calculated using the fundamental equation of population dynamics according to the 'Euler equation'. This equation is also referred to as 'Lotka equation':

$$\sum_{x=0}^{d} \mathrm{e}^{-r_m x} l_x m_x = 1$$

r_{m} = intrinsic rate of natural increase, x = time (days), l_x = age-specific survival probability, m_x = age-specific fecundity.

A Microsoft Visual Basic (6.0) macro provided by Daniel Muschiol, Department of Animal Ecology, University of Bielefeld, was used for r_{m} iteration. The intrinsic rate of natural increase (r_{m}) represents the theoretical growth rate of a population with stable age distribution in an unlimited environment. Population doubling time (PDT) was calculated according to the formula: PDT $= \ln 2/r_{\text{m}}$. Both, PDT and r_{m} are of theoretical nature, representing ideal growth conditions, which are usually not available.

Net reproductive rate and generation time was calculated according to the life table constructed with l_x and m_x (data not shown).

Net reproductive rate (R_0) depends on the age specific survival probability and fecundity, which is defined as the average number of juveniles that a female in a population produces during its life time: $R_0 = \sum l_x m_x$.

Alternative generation time was measured using the following equations (as described in Vranken & Heip, 1983): the age of the mother of an average newborn in an exponentially growing population (T): $T = \sum x \mathrm{e}^{-r_m x} l_x m_x$.

The Shapiro-Wilk test was performed to check the data for normal distribution. Homogeneity of variance was performed by the Fisher's F-test. Effects of different food densities on life cycle parameters were analysed using analysis of variance (ANOVA) and the Tukey's honestly significant differences (HSD) test. Student's t-test was performed to assess the significance of the difference between the means of two independent samples.

Results

Life history parameters obtained in hanging drop experiments with different cell density biomasses of *E. coli* and *C. cohnii* are presented in Tables 1 and 2, respectively. On *E. coli*, the life span of females ranged from 9 to 12 days (Table 1). No significant influence of different bacterial cell densities on the mean lifespan of females was recorded. Cultured on *E. coli* at a biomass of 335.63 μg ml^{-1} (3×10^9 cells ml^{-1}), the mean number of offspring was 214 individuals from each female (net reproductive rate, R_0). The R_0 was significantly higher at this density compared to all other densities (Tukey's HSD test, $P < 0.05$), hence, it can be considered closest to the optimum food density (Table 1). The time until the females reached the maximal reproduction rate (T_{rate}) was 5 days, compared with 6 or 7 at lower or higher densities, but these differences were not significant. Significantly lower generation time (T) with 5 days and population doubling time (PDT) with 0.7 days was recorded for the 335.63 μg ml^{-1} biomass. Accordingly, the intrinsic rate of natural increase (r_{m}) was the highest at 335.63 μg ml^{-1} and significantly different to values obtained at lower biomass. Maximum number of offspring produced by a single female varied between 342 (at 0.12 μg ml^{-1}) and 638 (at 335.63 μg ml^{-1}) for *E. coli* fed nematodes.

Table 2 represents the life history parameters of *Panagrolaimus* sp. fed with different cell density (biomass) of *C. cohnii*. At the highest biomass of 2478.82 μg ml^{-1}, the

Table 1. Life cycle parameters of *Panagrolaimus* sp. at different food densities of *Escherichia coli* at 27°C.

	Food density						$F_{5,12}$	P
Cell density (ml^{-1})	10^6	10^7	10^8	10^9	3×10^9	10^{10}		
Dry mass (μg ml^{-1})	0.12	1.12	11.19	111.88	335.63	1118.75		
N	109	85	106	96	107	97		
Lifespan (days)	12 ± 2.5^{a}	9 ± 0.7^{a}	10 ± 0.9^{a}	9 ± 0.3^{a}	11 ± 0.9^{a}	9 ± 0.4^{a}	3.0	0.05
R_0	80 ± 33.5^{a}	114 ± 27.5^{ab}	92 ± 26.7^{ab}	79 ± 13.7^{a}	214 ± 4.8^{c}	150 ± 12.8^{b}	16.7	<0.0001
Max. no. of offspring female^{-1}	342	346	330	279	638	372		
T_{rate} (days)	7 ± 0.1^{a}	6 ± 0.2^{b}	6 ± 0.3^{b}	6 ± 0.2^{b}	5 ± 0.4^{b}	6 ± 0.3^{b}	10.8	<0.001
T (days)	6 ± 0.4^{a}	6 ± 0.1^{ab}	6 ± 0.1^{abc}	6 ± 0.1^{bc}	5 ± 0.2^{c}	6 ± 0.3^{abc}	7.1	<0.001
r_{m} (day^{-1})	0.6 ± 0.1^{a}	0.7 ± 0.0^{ab}	0.7 ± 0.0^{ab}	0.7 ± 0.0^{ab}	0.9 ± 0.0^{c}	0.8 ± 0.0^{bc}	19.3	<0.0001
PDT (days)	1.1 ± 0.1^{a}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{ab}	0.7 ± 0.0^{c}	0.8 ± 0.0^{bc}	10.8	<0.0001

Values represent means ± SD. Within each row values with different superscripts are significantly different (post hoc Tukey's HSD test, $P \leqslant 0.05$). N = number of females observed; lifespan = mean number of days survived by the females; R_0 = net reproductive rate; T_{rate} = age in days at maximum rate of juvenile production; T = mean age of the mothers of a set of newborn individuals in a population with a stable age distribution; r_{m} = intrinsic rate of natural increase; PDT = population doubling time. F statistics and P values were calculated using ANOVA.

Table 2. Life cycle parameters of *Panagrolaimus* sp. at different food densities of *Crypthecodinium cohnii* at 27°C.

	Food density				$F_{3,8}$	P
Cell density (ml^{-1})	10^5	10^6	2×10^6	4×10^6		
Dry mass (μg ml^{-1})	61.971	619.711	1239.41	2478.82		
N	79	90	94	86		
Lifespan (days)	11 ± 0.8^{a}	12 ± 2.1^{a}	12 ± 0.3^{a}	12 ± 0.2^{a}	0.68	0.589
R_0	127 ± 11.4^{a}	140 ± 31.6^{a}	140 ± 1.8^{a}	216 ± 83.9^{a}	2.42	0.141
Max. no. of offspring female^{-1}	283	395	393	667		
T (days)	6 ± 0.3^{a}	6 ± 0.1^{a}	6 ± 0.2^{a}	6 ± 0.9^{a}	1.07	0.415
r_{m} (day^{-1})	0.7 ± 0.0^{a}	0.7 ± 0.0^{ab}	0.7 ± 0.0^{a}	0.8 ± 0.1^{b}	5.53	0.024
PDT (days)	1.0 ± 0.0^{a}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{a}	0.8 ± 0.1^{b}	5.9	0.020

Values represent means ± SD. Within each row values with different superscripts are significantly different (post hoc Tukey's HSD test, $P \leqslant 0.05$). N = number of females observed; lifespan = mean number of days survived by the females; R_0 = net reproductive rate; T = mean age of the mothers of a set of newborn individuals in a population with a stable age distribution; r_{m} = intrinsic rate of natural increase; PDT = population doubling time. F statistics and P values were calculated using ANOVA.

highest R_0 value (216; $P = 0.141$), the shortest time (6 days; $P = 0.082$) for maximum rate of juvenile production (T_{rate}), as well as the highest r_{m} (0.8; $P = 0.024$) and shortest population doubling time (PDT) with 0.8 ($P = 0.02$) days were recorded, although differences were not significant, except for r_{m} and PDT. Maximum number of offspring produced by a single female varied between 283 (at 61.971 μg ml^{-1}) and 667 (at 2478.82 μg ml^{-1}) algal biomass.

In general, at optimal food densities of *E. coli* or *C. cohnii* life-history trait parameters for *Panagrolaimus* sp. (strain NFS 24-5) did not differ (Student's t-test, $P = 0.87$). With both types of food source, highest net reproductive rate (R_0) was comparable, but this yield was obtained on more than 7× lower biomass of *E. coli*. Also, lowest T_{rate} and shortest generation time (T) were 1 day earlier when the nematodes were fed with *E. coli*. Highest value of r_{m} and shortest PDT value was 0.9 and 0.7, respectively, with *E. coli* and 0.8 with *C. cohnii*.

Discussion

Production of nematodes for use as living food for aquaculture organisms requires a strain, which can produce high numbers of offspring within a short process time. *Panagrolaimus* sp. (strain NFS 24-5) is compared to

other investigated nematodes of the same genus, the one with the shortest observed generation time and highest reproduction rate (Ayub *et al.*, 2013a). Strain NFS 24-5 thus has a comparatively high potential for mass production.

Nearly the same life history parameters were achieved when nematodes were fed with *E. coli* and *C. cohnii* at the most suitable food density tested. This indicates that strain NFS 24-5 is a generalist with low feeding selectivity and that higher offspring numbers and lower generation times might not be obtainable with this strain. However, the results of this investigation also indicate that *C. cohnii* is less effective as food source in mass production of *Panagrolaimus* sp. strain NFS 24-5 than *E. coli*. A much higher biomass of *C. cohnii* is required than with *E. coli* to reach comparable offspring numbers. The reason might be the size of dinoflagellate cells: *C. cohnii* forms two types of cells, swimming cells and cysts (Bhaud *et al.*, 1991). In the hanging drop experiments swimming cells of 5-10 μm in size were used to feed the adult nematodes. These swimming cells have only a thin and flexible theca (Mendes *et al.*, 2009). The buccal cavity of the adult nematodes of NFS 24-5 was measured as 4.5 μm (mean of 25 individuals), indicating that these nematodes could only feed on the smaller cells of about 5 μm diam. Optical investigation of *C. cohnii*-fed nematodes clearly indicated that cells of the dinoflagellates had been ingested. The diameter of the buccal cavity does not necessarily limit the diameter of the food source for nematodes, because flexible cells can be sucked into the mouth cavity even if they have a slightly larger diameter (Moens *et al.*, 2006). However, much bigger cells might not be ingestible and, therefore, most of the available biomass in the hanging drops could not be used by the nematodes. Hence, *E. coli* is more appropriate as food source in mass production as the size of bacteria is usually between 1-4 μm in length and 0.5-1.3 μm in width (Salinas *et al.*, 2007).

The other major objective was to investigate the influence of variable cell density on the reproductive potential of the female nematode. The results clearly indicate that a cell density of *E. coli* exceeding 3×10^9 is beyond the optimal food supply. The reason for such a decline in offspring production is not known. It might be that oxygen supply becomes a limiting factor at such high bacterial densities. On the other hand, it had been shown earlier that bacteriophagous nematodes have a certain optimum for the bacterial density regarding the food absorption rate, which reduces when the optimal density is exceeded (Moens & Vincx, 2006; Santos *et al.*, 2008). This leads to reduced offspring production and lower intrinsic growth rates (r_m).

Escherichia coli grows faster than *C. cohnii* and it provides large numbers of nematode offspring within a shorter time, as the generation time and population doubling time is lower with *E. coli* than with *C. cohnii*. The mass production process must therefore be divided into two steps, the mass production on *E. coli* and the subsequent enrichment of the nematodes with fatty acids on *C. cohnii*. If the nematode product contains many J1, disrupting *C. cohnii* cells must be taken into consideration. Ideally, the flagellates ingestion rate by the nematode is improved when disrupted cells are provided. Another possibility could be to grow nematode on a mixed diet of *E. coli* and disrupted *C. cohnii* cells. Then, specific research should be conducted on the mixed diet to determine the optimum density.

Whether results from hanging drop experiments can provide information on the necessary cell density in *in vitro* liquid culture production needs further investigation. In hanging drops, two adult nematodes are transferred daily to fresh cells always at the same density. The nematode density is in 10 μl is 2. In liquid batch cultures bacterial density is not directly adjustable and variable. Nematodes are added at the earliest 1 day after inoculation of the bacteria at much higher densities between 1000 and 5000 ml^{-1} and as nematodes start feeding the bacterial density is constantly declining (Ehlers, 2001). Whether similar yields per female recorded during this investigation will be reached in batch liquid cultures will also have to be investigated. A maximum yield of 238 000 ml^{-1} has been reported for NFS 24-5 cultured on yeast cells at an inoculum density of 4000 nematodes ml^{-1} (Honnens & Ehlers, 2013a). Considering a sex ratio of 1:1 and the maximum offspring potential of 214 per female at a bacterial density of 3×10^9 up to 426 000 nematodes ml^{-1} could be expected ideally after one generation. However, the hanging drop methods provides a constant supply of high bacterial cell density at lower nematode densities. Consequently, so far only significantly smaller amounts of nematodes (max. 251 000 ml^{-1}) could be harvested in liquid cultures of NFS 24-5, as shown by Ayub *et al.* (2013b).

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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Monoxenic liquid culture with *Escherichia coli* of the free-living nematode *Panagrolaimus* sp. (strain NFS 24-5), a potential live food candidate for marine fish and shrimp larvae

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Abstract The free-living, bacterial-feeding nematode *Panagrolaimus* sp. (strain NFS 24-5) has potential for use as live food for marine shrimp and fish larvae. Mass production in liquid culture is a prerequisite for its commercial exploitation. *Panagrolaimus* sp. was propagated in monoxenic liquid culture on *Escherichia coli* and parameters, like nematode density, population dynamics and biomass were recorded and compared with life history table data. A mean maximum nematode density of 174,278 mL^{-1} and a maximum of 251,000 mL^{-1} were recorded on day 17 after inoculation. Highest average biomass was 40 g L^{-1} at day 13. The comparison with life history table data indicated that the hypothetical potential of liquid culture is much higher than documented during this investigation. Nematode development is delayed in liquid culture and egg production per female is more than five times lower than reported from life history trait analysis. The latter assessed a nematode generation time of 7.1 days, whereas the process time at maximum nematode density in liquid culture was 16 days indicating that a reduction of the process time can be achieved by further investigating the influence of nematode inoculum density on population development. The results challenge future research to reduce process time and variability and improve population dynamics also during scale-up of the liquid culture process.

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Keywords Aquaculture · *Escherichia coli* · Population dynamics · Life history table · Biomass · Nematode density · Offspring production

Introduction

First feeding of marine fish and shrimp larvae is challenging, as larvae are fragile, the digestive system is not yet fully developed and major morphological and physiological changes occur during this developmental stage (Lavens and Sorgeloos 2000). Hatcheries producing larvae rely on live prey, like rotifers or the brine shrimp *Artemia salina*, which are expensive to produce, often vary in quality (Lavens and Sorgeloos 2000) and can carry opportunistic and potentially pathogenic microorganisms deleterious for delicate early larval stages (Skjermo and Vadstein 1993; Haché and Plante 2011; Seychelles et al. 2011). As marine larvae require essential polyunsaturated fatty acids, live food organisms must be enriched prior to larval feeding. During fatty acids enrichment, the load with bacteria or fungi can further increase (Høj et al. 2009; Seychelles et al. 2011, 2013). In order to reduce dependence on these organisms and provide food organisms free of contaminants, research has concentrated on the use of free-living, microbivorous nematodes (Biedenbach et al. 1989; Kumlu et al. 1998; Schlechtriem et al. 2005). Nematodes can be cultivated under sterile, monoxenic conditions (Ehlers 2001). Recent research has concentrated on the nematode *Panagrolaimus* sp. strain NFS 24-5 (Honnens and Ehlers 2013a) because it can survive under anhydrobiotic conditions, which makes storage and transportation easier (Honnens et al. 2013a) and because it can be enriched with essential fatty acids (Honnens et al. 2013b)

To be able to provide hatcheries with nematodes, techniques for cost-effective mass production, must be available

(Ehlers 2001). Honnens and Ehlers (2013a) were the first to attempt liquid culture of the free-living *Panagrolaimus* sp. (strain NFS 24-5). They used baker's yeast, *Saccharomyces cerevisiae* as food supply. After a lag phase of up to 8 days, adult nematodes started to produce offspring. Maximum yields were highly variable between culture flasks. Thirteen to 15 days after nematode inoculation, nematode density was between 45 and 238×10^3 mL^{-1}. Attempts to grow the nematodes in 20-L bioreactors (Honnens and Ehlers 2013b) were successful and the lag phase was reduced to 4 days. Mean yields were at 128 ± 79 and maximum at 270×10^3 nematodes mL^{-1}. Fed-batch condition could neither reduce variability nor increase the yields (on average of $63\pm39\times10^3$ nematodes mL^{-1}).

To be able to better understand the basic developmental and reproductive biology of the nematode *Panagrolaimus* sp. NFS 24-5 and conclude on the possible potential of liquid culture production, Ayub et al. (2013) cultured pairs of single male and female in hanging drops of *Escherichia coli* cell suspension with a density of 3×10^9 mL^{-1} and transferred the nematodes to fresh drops every day to provide food ad libitum. Life history traits and growth parameters, for instance, nematode's age at sexual maturity, lifespan, net reproductive rate, total fertility rate, generation time, intrinsic rate of natural increase, population doubling time and somatic growth rate, were assessed.

The present study cultured the nematodes *Panagrolaimus* sp. strain NFS 24-5 in monoxenic liquid culture on *E. coli* and compared the results with life history trait data obtained from a previous investigation (Ayub et al. 2013).

Materials and methods

Bacterial stock cultures

E. coli (strain K12, DSM 498) was used to culture *Panagrolaimus* sp. (strain NFS 24-5). The bacteria were incubated in sterile 100-mL Erlenmeyer flask containing 25 mL LB5 medium (10 g trypton, 5 g yeast extract, 5 g NaCl, and 1 L distilled water) for 24 h at 25 °C on a rotary shaker at 200 rpm. The bacterial suspension was mixed with glycerol to reach 15 % (*v*/*v*) and stored at −80 °C in 2-mL Eppendorf tubes until use.

Establishment of monoxenic nematode liquid cultures

Monoxenic cultures of *Panagrolaimus* sp. (strain NFS 24-5) were established according to the method described in Ayub et al. (2013). Hatching bacteria-free first juvenile stages (J1) obtained from surface-sterilized eggs were transferred to nematode growth medium containing 3 g NaCl, 17 g agar, 2.5 g soy peptone, and 975 mL distilled water seeded with *E. coli*. Agar plates (9.2 cm diameter) were incubated at 25 °C for 5 days. Afterwards, one agar plate was used to inoculate one 250-mL flask containing 50 mL OM4 medium (2 g glucose, 4 g yeast extract, 7 defatted soy flour, 3 g lecithin, 30 g rapeseed oil, 5 g NaCl, 1 g KCl, 0.5 g $CaCl_2$, 1 g $MgSO_4\times7$ H_2O, 3.4 KH_2PO_4, 1.7 g K_2HPO_4, and 1 L distilled water). The OM4 medium had been inoculated 1 day before with 3 % (*v*/*v*) of a 24-h-old *E. coli* preculture in OM4 medium, which had been started from frozen stocks. These liquid cultures were further subcultured to produce nematode inoculum for the experiments.

Nematode liquid culture experiment

E. coli was precultured in OM4 medium for 48 h and then nematodes from monoxenic liquid cultures were inoculated at a density of 5,000 mL^{-1}. The experiment was carried out three times with six 250-mL flasks containing 50 mL liquid medium for each replicate. To provide homogeneous condition, all flasks of one replicate were started from a single 24-h-old *E. coli* culture. After another 24 h, *Panagrolaimus* sp. were inoculated also from a single monoxenic liquid preculture. An aliquot of about 1 mL culture medium from each culture flask was withdrawn every second day (until the total nematode density started to decrease) and the bacterial and nematode density and nematode length measurement were assessed.

Assessment of bacterial and nematode density

Samples were mixed and an aliquot of 100 μL was taken, mixed with 900 μl 10 % Histofix (Roth, Karlsruhe, Germany). Sample size was reduced to 400 μL when the bacterial density decreased. Bacterial cell density was assessed using a Thoma chamber (0.01 mm depth, Poly Optic GmbH) for cell counts. For each flask, two subsequent samples were assessed and each sample was counted twice. To count nematodes, samples were diluted to reach 50–200 nematodes per chamber of a cloning plate with a grid of 16 squares (Bio-one Cellstar® No. 704160; Greiner, Solingen, Germany) and assessed under an inverted microscope (Axiovert 25, Zeiss, Germany) at 50-fold magnification.

Nematode total length measurement and biomass

For length measurements, nematodes were heat killed at 70 °C and stored at −20 °C until measured under a dissection microscope (Zeiss, Stemi SV11, West Germany) with the aid of an attached camera (Sony, Color-view) at 32-fold magnification. The image analysis software analySIS® (Soft Imaging System GmbH, Germany) was used. Biomass per

individual was calculated according to the formula of Andrassy (1956),

$$B = \frac{W^2 \times L}{16 \times 10^5}$$

where, "w" is the maximum body width in micrometer, "L" is the total length in micrometer, and "B" is the biomass per individual in microgram (fresh weight).

Maximum body width was calculated by using a cubic polynomial model described by Honnens and Ehlers (2013a) for the same *Panagrolaimus* sp. strain. The average biomass per individual was multiplied by the total number of nematodes per milliliter. The number of nematodes measured per sample varied from 100 to 168. The lengths of measured nematodes ranged from 80 to 1,382 μm.

Modeling the hypothetical nematode population development

Data obtained on the population development under unlimited food supply (Ayub et al. 2013) were used to model the possible offspring production and composition of the population in liquid culture with an inoculum density of 5,000 nematodes mL^{-1} consisting of J1 larva. The sex ratio was considered to be 1:1. Observations by Ayub et al. (2013) document a development from J1 to adults in 4 days. From that day onwards, the number of 2,500 females per milliliter was multiplied with the mean number of daily offspring production and the resulting number was added to the total number of offspring of the previous day. The size class distribution of each day was calculated on the basis of the individual growth data (Ayub et al. 2013). One day is necessary for development from freshly hatched juveniles to the middle size class (400–800 μm) and another to 2 days to grow to the biggest size class (>800 μm). Nematode biomass was calculated according to the same formula applied in the present study for liquid cultures.

Statistics

The net offspring production per female of a given day was calculated as follows: $(n_{d+2}-n_d)/2/f_d$ (n=total number of nematodes, f=number of females=50 % of total adults, d=day, sampling was done only every second day). One-way analysis of variance (ANOVA) was performed to compare the data on nematode maximum density, total biomass, and biomass excluding adults between the three replicates. Shapiro–Wilk test was performed to test the normal distribution of the data. A nonparametric test, Kruskal–Wallis one-way analysis of variance on rank test was performed in case the normality test failed. Pearson's correlation coefficient (r) was calculated to measure the strength of association between two variables.

Results

No significant differences were observed among all three replicates of the experiment for the parameters: maximal nematode density (ANOVA: df=2, 17; F=1.354; p=0.288), maximal biomass in total (ANOVA: df=2, 17; F=0.0427; p=0.958) and maximal biomass excluding adults (ANOVA: df=2, 17; F=3.179; p=0.071). The population development recorded in all 18 flask cultures were therefore used to calculate means (Fig. 1).

The initial mean *E. coli* density was 83×10^9 cells mL^{-1} (Fig. 1a). It continuously declined until the end of the experiment. Minimum and maximum cell density values for *E. coli* at each sampling day are presented in Table 1. The severe

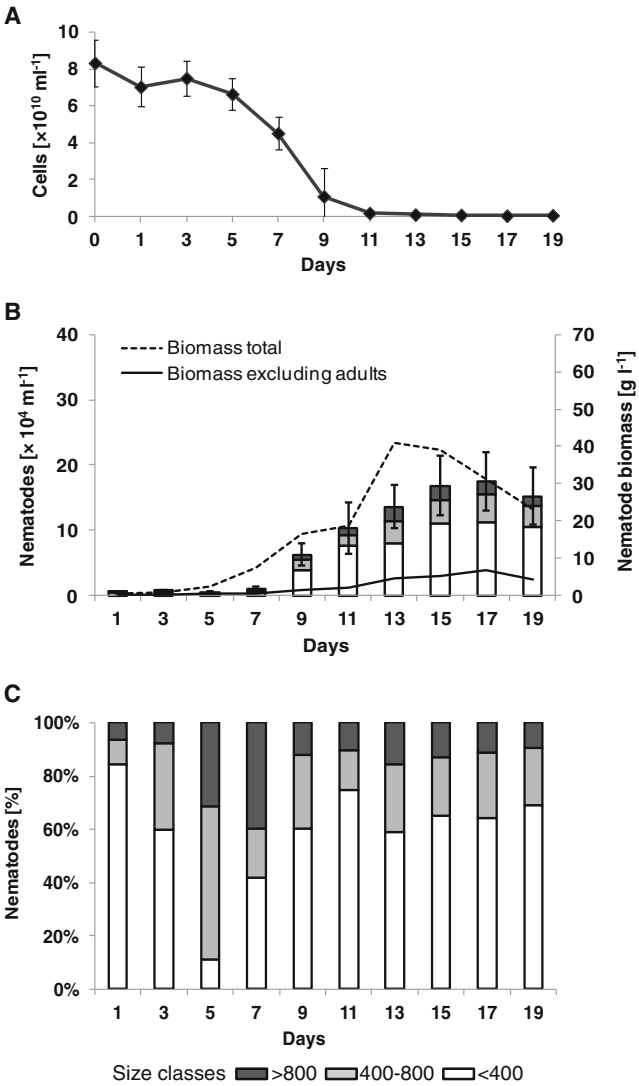

Fig. 1 Mean population dynamics of *Panagrolaimus* sp. strain NFS 24-5 in liquid medium with *E. coli* at 25 °C. **a** *E. coli* cell density [$\times10^{10}$ mL^{-1}], **b** nematode density [$\times10^4$ mL^{-1}] in three size classes (micrometer) and nematode biomass [gram per liter], **c** percentages of three nematode size classes. *Error bars* standard deviation of the mean (n=18)

Table 1 Minimum and maximum *E. coli* and *Panagrolaimus* sp. (NFS 24-5) densities, nematode lengths, and standard deviations in liquid culture at 25 °C (n=18). Mean bacterial and nematode densities per day is presented in Fig. 1

	Bacterial density [$\times 10^9$ mL^{-1}]			Nematode length [μm]		Nematode density [$\times 10^3$ ind. mL^{-1}]		
Days	Min	Max	SD	Min	Max	Min	Max	SD
0	47.00	99.00	12.81					
1	51.00	87.00	10.70	90	1,356	3.05	6.65	1.98
3	56.00	92.00	9.35	94	1,291	1.80	7.85	1.51
5	51.00	80.00	8.44	105	1,313	0.65	7.05	1.31
7	33.00	63.00	8.94	96	1,382	2.10	19.60	2.54
9	1.60	43.00	15.36	93	1,337	13.60	88.00	17.22
11	0.92	8.35	1.80	83	1,323	24.00	172.50	38.72
13	0.80	1.40	0.18	81	1,358	66.00	200.00	33.33
15	0.65	1.00	0.10	79	1,259	103.00	241.00	45.74
17	0.18	0.95	0.21	80	1,307	105.00	251.00	44.88
19	0.40	1.00	0.15	80	1,264	75.00	208.00	44.01

decline in cell counts coincides with the occurrence of the maximum percentage of adults on day 7 (Fig. 1c).

Flasks were inoculated at a density of approximately 5,000 nematodes mL^{-1} with >80 % of small juvenile stages (Fig. 1c). The average nematode density reached a minimum at day 5 with 4,739 mL^{-1} (Fig. 1b). Inoculated adults counted on days 1 and 3 did either not contributed to offspring production or J1 mortality was counteracting a population increase during the first culture days as only a minor increase in population density from day 1 (5,283 mL^{-1}) to day 3 (5,997 mL^{-1}) was observed. Data on minimum length confirm the continuous presence of freshly hatched juveniles of approximately 80–90 μm as well as adult stages >1,200 μm (Table 1). The mean percentage of pre-adult juveniles (400–800 μm) increased continuously until day 5 and the proportion of adults reached the maximum at day 7 (Fig. 1c). These pre-adults and adults developed from the inoculated juveniles. Average nematode density increased more than double between days 5 and 7 (4,739–10,878), indicating offspring production by the adults counted on day 5. A day after a major proportion of adults was recorded (Fig. 1c), an abrupt rise in juvenile stages at day 9 was assessed. The mean population density increased until day 17 (174,278 nematodes mL^{-1}) and then started to decrease.

A continuous increase in average total nematode biomass (Fig. 1b) was observed from the beginning of the culture period until day 11. A threefold increase of biomass was recorded from days 3 to 5 (0.71–2.33 g L^{-1}) and from days 5 to 7 (2.33–7.4 g L^{-1}; Table 2), which is due to the increase in adults (Fig. 1c). From days 11 to 13, the total biomass increased more than double (from 18 to 40 g L^{-1}), which was the highest value recorded. Afterwards, biomass decreased continuously until the end of the culture period. The minimum and maximum total biomass and biomass excluding adults (Table 2) also indicates a continuous increase in biomass. The highest biomass value of 88 g L^{-1} was recorded on day 17.

Biomass excluding adults was recorded as well because adults might be too long and thus not suitable for feeding (Lavens and Sorgeloos 2000). Their number is not exceeding 10 % at the end of the process and would therefore only be of minor importance for the shrimp and fish larval diet. A continuous, but less dramatic increase in biomass is observed until day 17. Obviously, few adults largely increase the total biomass. Of a total maximum biomass of 88 g L^{-1}, non-adult stages only contribute with 14 g L^{-1} to the total value (Table 2).

A hypothetical nematode population development, calculated based on life table data obtained from observations of several pairs of male and female nematodes in hanging drops of 3×10^9 cells of *E. coli* (Ayub et al. 2013) is presented in Fig. 2. Based on these fundamental biological data and the inoculum density

Table 2 Minimum and maximum and standard deviation of total biomass and biomass excluding adult nematodes of length >800 μm of *Panagrolaimus* sp. (NFS 24-5) recorded from in liquid culture at 25 °C (n=18). Mean nematode biomass per day is presented in Fig. 1

	Nematode biomass [g L^{-1}]					
	Biomass total			Biomass excluding adults		
Days	Min	Max	SD	Min	Max	SD
1	0.23	1.22	0.28	0.03	0.18	0.05
3	0.25	1.53	0.32	0.12	0.33	0.07
5	0.47	6.61	1.76	0.04	0.83	0.20
7	1.38	13.28	3.63	0.11	1.51	0.36
9	5.19	28.12	6.64	0.30	3.48	0.82
11	8.61	42.38	8.63	0.97	3.41	0.74
13	21.50	71.46	16.20	2.24	7.94	1.82
15	15.28	69.50	15.47	2.66	8.93	1.96
17	9.67	88.18	17.85	2.72	14.00	3.06
19	5.57	73.95	16.45	2.23	8.39	1.76

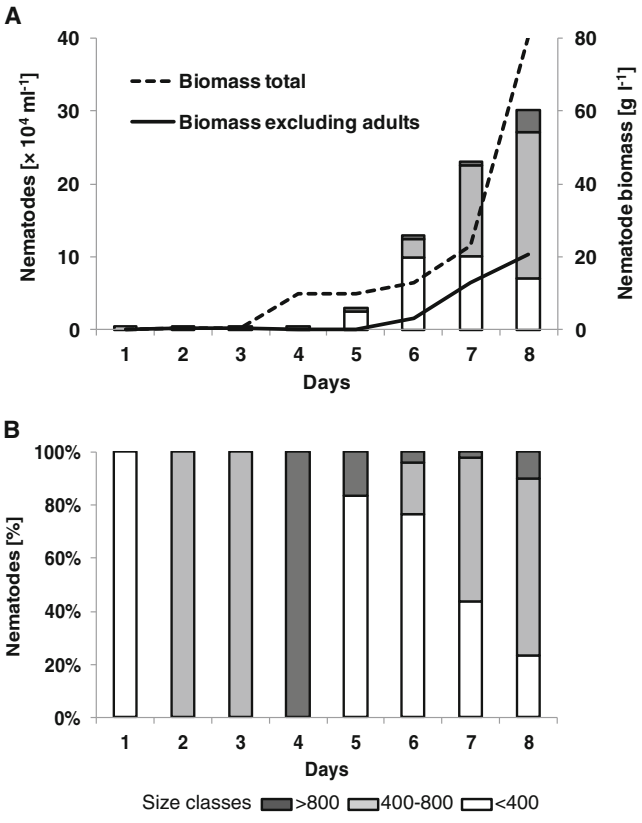

Fig. 2 Hypothetical population dynamics of *Panagrolaimus* sp. strain NFS 24-5 calculated from life history trait data in hanging drops at 25 °C with *E. coli* as food source. Life table data were obtained from Ayub et al. (2013). **a** Nematode density [$\times 10^4$ mL^{-1}] in three size classes (micrometer) and nematode biomass [gram per liter], **b** percentages of three nematode size classes (micrometer)

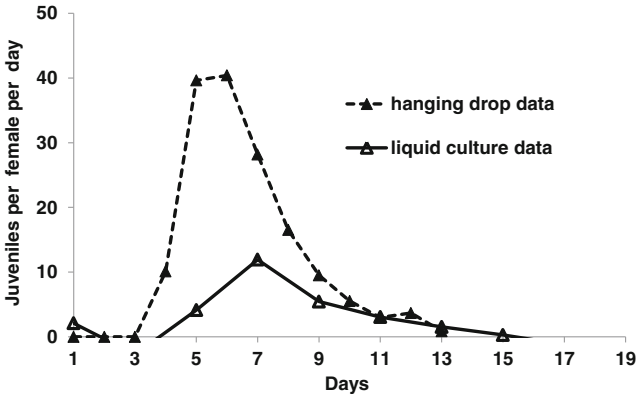

Fig. 3 Mean daily net offspring production per female nematode in hanging drop (n=91 females observed) and liquid culture (n=18 flask cultures) at 25 °C with *E. coli* as food source. Data from hanging drops were obtained from Ayub et al. (2013)

used for liquid culture experiments, we would expect a population density increase already on day 5 and within 8 days the density would reach 297,500 nematodes mL^{-1} under the assumption of unlimited food supply. J1 need 3 days to develop into pre-adult stages. They developed into adult stage at day 4 and start to lay eggs at the same day. After 24 h, J1 hatch (Ayub et al. 2013). No increase in population density can thus be expected within this time period (Fig. 2a and b) in case a liquid culture is inoculated only with J1. Biomass would start to increase at day 4.

Daily net offspring production per female in hanging drop and in liquid culture is presented in Fig. 3. The net offspring production includes daily offspring production and mortality. Females produced an average of 40 juveniles at days 5 and 6 in hanging drop under unlimited food supply (Ayub et al. 2013). No juvenile mortality was observed under those conditions; however, hatching rate from eggs was not assessed. Maximum net offspring production in liquid batch culture was only 12 per female at day 7. The number of dead animals was not assessed. From days 3–5 to 17–19, the mortality exceeded offspring production as it can be seen in Fig. 1. The total number of offspring per female was 28 in liquid culture and 156 in hanging drops (Ayub et al. 2013).

The correlation between bacterial density in the first week and maximum nematode density and between nematode density in the first week and maximum nematode density in each flask was calculated from liquid culture data (Table 3). The maximal nematode density was significantly positively correlated with the inoculated bacterial density at day 0 and with the nematode density on days 3–7. The correlation coefficient between the number of nematodes in the first week and the maximal nematode density is increasing from days 1 to 7 (Table 3). On the other hand, the maximum nematode density was significantly negatively correlated with the bacterial density at day 7. This is due to the fact that the bacterial density at day 7 is significant negatively correlated with the number of adult nematodes on this day (Pearson r=−0.493, p=0.038), which means that increasing numbers of adults significantly reduce the number of bacteria. However, the maximal nematode density is positively correlated with the number of adults at day 7 (Pearson r=0.734, p=0.001).

Table 3 Correlation between initial bacterial density and nematode densities during the first week of the liquid culture process and nematode inoculation density and maximum density of flask cultures at 25 °C (n=18)

	Pearson correlation coefficient			
Days	Bacterial density versus maximum nematode density	p	Nematode density versus maximum nematode density	p
0	0.491	0.038		
1	−0.076	0.764	0.343	0.118
3	−0.007	0.977	0.572	0.013
5	−0.005	0.984	0.657	0.003
7	−0.477	0.045	0.710	0.001

Discussion

Nematode production in liquid culture

Taking into consideration maximum nematode yields of $>25\times10^4$, an increase of mean yields of *Panagrolaimus* sp. liquid culture may be possible and if these numbers can also be realized during process scale up the mass production of this nematode for use as food in hatcheries for marine larval shrimps and fish might be interesting for commercial exploitation. However, the process time of 15–17 days is still too long. Improvements seem to be possible as the hypothetical population dynamics indicate that maximum yields should be possible to reach within 8 days. The question is how to reach this goal?

Increase of nematode inoculation density is a parameter worth to be further investigated. Honnens and Ehlers (2013a, b) cultured *Panagrolaimus* sp. strain NFS 24-5 in liquid medium using yeast, *Saccharomyces cerevisae*, as food organism. Compared to data obtained with *E. coli* as food source (mean maximum nematode density of 174,278 mL^{-1} and maximum of 251,000 mL^{-1}), yield was lower with yeast (119.236 and 237,000 mL^{-1}, respectively), but the differences are remote. What is more interesting is that Honnens and Ehlers (2013a, b) carried out two experiments with nematode inoculum densities of 3,000 and 4,000 mL^{-1}. Highest nematode densities were obtained with the higher nematode inoculum (mean maximum 135,620 mL^{-1}) compared to the lower inoculum density (102,850 mL^{-1}). The inoculum density in the presented experiments ranged between 3,050 and 6,650 mL^{-1} and was positively correlated (first week counts) with the maximal nematode density in a culture. The higher nematode inoculum density could be one reason why higher maximal nematode densities could be obtained in our experiments compared to Honnens and Ehlers (2013a, b). Increase in nematode inoculum density thus may also be one parameter which could have potential for further process improvement.

Density of the microorganisms used to feed the nematodes might be another parameter for improvement. Although no clear influence of the yeast density on maximal nematode yields were reported by Honnens and Ehlers (2013a, b), in their experiment with the higher nematode inoculum the yeast density was only 50 % of that used in the experiment with lower nematode density. However, in the cultures with the lower yeast density, higher amounts of nematodes were observed and it is not clear whether the reason was the lower yeast density or the higher nematode inoculum. In the presented experiments, *E. coli* density at the time of nematode inoculation was positively correlated with the maximal nematode density.

The high variability also excludes the current process technology from commercial use. Maximum nematode yields ranged from 111,000 to 251,000 mL^{-1} on *E. coli* and from 45,000 to 238,000 in the yeast cultures (Honnens and Ehlers 2013a, b). Whether this variation is caused by the variable nematode and bacterium inoculum densities needs further investigation.

Comparison between hypothetical population growth with unlimited food and liquid batch culture

Whether nematode yields recorded per female under unlimited food supply in a hanging drop and whether life history table data (Ayub et al. 2013) can be used to predict yields in liquid culture is, of course, questionable. However, the comparison stimulates discussion and provides possible approaches for improvement. A delay of development of 1 day during the early phase of liquid culture becomes obvious when comparing liquid culture population development with the hypothetical nematode development based on observations in hanging drops. One reason might be that nematodes need longer to adapt to liquid culture conditions. The bacterial density in liquid culture is more than 10 times higher than those used in hanging drops (Ayub et al. 2013). Ayub et al. (unpublished) investigated the optimum *E. coli* cell density in hanging drops. At 10^{10} cells mL^{-1}, the generation time was delayed by 1 day in hanging drops. On the other hand, a significant positive correlation between the bacterial cell density and the maximal nematode densities was calculated. Thus, a high bacterial density may hamper individual growth in the beginning, but also support offspring production later during the process.

More important than a 1-day delay during the early phase of liquid culture is the eminent delay in offspring production (increase in J1 on day 9 instead of day 5) and the considerable reduction in offspring per female, which was 5.5 times lower in liquid culture than recorded in hanging drops. The maximum number of offspring per female (12) was produced on day 7 in liquid culture. In hanging drops, 40 offspring per female were observed between days 5 and 6. In liquid culture, offspring production is scattered over a period of 15 days, in hanging drops egg laying ceases after 13 days. This difference is not very pronounced. Consequently, the focus needs to concentrate on the question, why young females in liquid culture lay so few eggs. Limited food supply in liquid culture can be excluded as the cell density was higher than in hanging drops. One possibility is that turbulent flow in liquid cultures reduced copulation and fertilization of eggs another is that J1 might have died. These points also need further attention during future investigations.

Ayub et al. (2013) reported a generation time of approximately 7 days and a life span at which 50 % of the reproductive females have died of 10 days. The time to obtain maximum yields in liquid culture would allow the development of two generations. Due to the low offspring production in the first generation, several juveniles developed further to adults and yields obtained at day 17 probably comprise of offspring

of both generations. Lower bacterial density available during the second nematode generation has resulted in lower number of eggs. Also, older females lay fewer eggs per day and egg laying finally ceases. The age of the females in preculture that were inoculated to the flasks was 13 days, which is the reason why these adults did not contribute to offspring production. In liquid culture, first-generation adults, which had developed from inoculated juvenile stages, should have ceased egg laying by day 17, which coincides with the decline in the total population density. At that day, the bacterial density reached 6×10^8 mL^{-1}. May be this was insufficient for J1 stages to survive as well. Schiemer (1982) also reported high larval mortality below a cell density of 10^9 mL^{-1} of *E. coli* for the rhabditid nematode, *Caenorhabditis briggsae*, which support this assumption.

Total number of nematodes is an important parameter, however, more important for feeding marine larval stages will be the biomass (Honnens and Ehlers 2013a, b). Adults contribute most to biomass, but may not be the optimal food, as the mouth size of first-feeding larvae usually mechanically restricts the size of the food particles which can be ingested. For example, at first-feeding, salmonids are able to consume particles as large as 1,000 μm, compared with only 100 μm in the case of first-feeding Gilthead seabream larvae (Lavens and Sorgeloos, 1996). Our nematodes have a size range between 80 and 1,382 μm, thus another parameter to be considered and room for improvement of population development.

In summary, much research is still needed to reduce process time and variability and improve population dynamics also during scale-up of the liquid culture process.

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