

Methodology article

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Chemically defined medium and *Caenorhabditis elegans*

Nathaniel J Szewczyk, Elena Kozak and Catharine A Conley*

Address: Space Life Research, NASA Ames Research Center, Moffett Field, CA 94035-1000, USA

Email: Nathaniel J Szewczyk - nate@alumni.cmu.edu; Elena Kozak - ekozak@mail.arc.nasa.gov; Catharine A Conley* - Cassie.Conley@nasa.gov

* Corresponding author

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Abstract

Background: *C. elegans* has been established as a powerful genetic system. Use of a chemically defined medium (*C. elegans* Maintenance Medium (CeMM)) now allows standardization and systematic manipulation of the nutrients that animals receive. Liquid cultivation allows automated culturing and experimentation and should be of use in large-scale growth and screening of animals.

Results: We find that CeMM is versatile and culturing is simple. CeMM can be used in a solid or liquid state, it can be stored unused for at least a year, unattended actively growing cultures may be maintained longer than with standard techniques, and standard *C. elegans* protocols work well with animals grown in defined medium. We also find that there are caveats to using defined medium. Animals in defined medium grow more slowly than on standard medium, appear to display adaptation to the defined medium, and display altered growth rates as they change the composition of the defined medium.

Conclusions: As was suggested with the introduction of *C. elegans* as a potential genetic system, use of defined medium with *C. elegans* should prove a powerful tool.

Background

Cultivation in chemically defined media was promoted at the initial suggestion of the utility of *C. elegans* as a genetic model system [1]. However, the development of a defined medium (*C. elegans* Maintenance Medium (CeMM), [2]) lagged far behind the development of *C. elegans* as a genetic system [3]. As a result monoxenic cultivation of *C. elegans* on Nematode Growth Medium agar plates with *E. coli* (NGM) is standard [4]. The ability to alter systematically the composition of CeMM should make it a staple in metabolism and aging studies. Additionally, cultivation using CeMM now allows removal of the variables introduced by monoxenic cultivation. Some of these variables impact nematode biology [5], with reduction of lifespan being the impact most recently demonstrated [6]. Monoxenic cultivation also introduces variables into pharmaceutical studies on *C. elegans* [4,7], an example being lack of

drug effect being due to *E. coli* metabolizing the drug. Large scale screening of pharmaceutical and nematicidal compounds on *C. elegans* can now be achieved when liquid CeMM is used with equipment for automated culturing [8] and experimentation ([9], updated version available from Union Biometrica). CeMM may also be useful in growing large numbers of animals for genomic or proteomic work and in development of *C. elegans* biosensors. We made arrangements with Fisher to have a 2x stock of CeMM produced by MediaTech. We provided Fisher a protocol (see Additional file 1) adapted from Lu and Goetsch [2]. All studies presented here utilized the same batch, though growth rates did not vary between two batches.

Table 1: Equipment compatible with *C. elegans* in liquid CeMM

<u>Material:</u>	<u>Manufacturer:</u>
<u>Tubes:</u>	
1 ml	Eppendorf
15 ml conical	Falcon
50 ml conical	Falcon
<u>Dishes:</u>	
60 mm petri	Falcon
100 mm petri	Falcon
60 mm tissue culture	Falcon
100 mm tissue culture	Falcon
24 well tissue culture	Corning
96 well tissue culture	Corning
<u>Flasks:</u>	
25 cm ² cell culture	Corning
75 cm ² cell culture	Corning
<u>Membranous devices:</u>	
10 mm membrane tubing	Spectrapor
Opticell™	BioCrystal
FEP bag	American Fluoroseal

Results and Discussion

Growing *C. elegans* in liquid CeMM

Actively growing cultures may be maintained longer using CeMM than NGM. Growth arrested L1s and dauers are observed after approximately one week on NGM plates, whereas healthy, reproducing populations were observed for at least one week, at room temperature, in unchanged liquid CeMM, in all tested materials (Table 1). Healthy, reproducing populations were observed for at least one month in the flasks, Opticells™, and FEP bags. With small initial populations healthy, reproducing animals were observed after two or three months on 1.7% agar CeMM plates or in unchanged liquid CeMM.

Significant evaporation of liquid CeMM was noted in 75 cm² flasks and 10 mm tubing. In 10 mm tubing, a starting volume of 1 ml of worms in liquid CeMM completely evaporated in seven days when sealed in a 100 mm petri dish or in six days when sealed in a Kennedy Space Center fixation tube (KFT [10]). Inclusion of a moist paper towel inside a 100 mm petri dish or a KFT allowed growth for 22 days with 300 μl of liquid CeMM remaining, a loss of two thirds the starting volume of CeMM. In contrast, evaporation of two-thirds of the starting volume of liquid CeMM in 75 cm² flasks is observed to take four to five weeks. While not extensively quantitated, evaporation does occur in other culture devices. The Opticell™ or FEP bag allowed adequate oxygenation of stationary cultures and appeared to minimize evaporation. After nine months a FEP bag contained 8 ml of the 11 ml starting culture volume of liquid CeMM. We presume animal movement was sufficient to mix liquid CeMM, if indeed mixing CeMM is necessary

to dilute wastes and evenly distribute nutrients. Use of either device also facilitated transport and shipment of animals. More animals were observed to settle to the bottom of the culture in FEP bags than in Opticells™, but sedimentation did not have obvious effects on population growth or health. Sedimentation appears to be the result of increased depth of the culture surface and lack of FEP bag rigidity versus the Opticell™. These results demonstrate CeMM can easily be used with a variety of laboratory devices and that CeMM grown animals require less maintenance than NGM grown animals.

In all devices, we often noted clumps of eggs in the medium, perhaps due to self-association, ruptured adult animals, or both. As many as 80% of these eggs failed to develop when clumps were placed in fresh liquid CeMM. This is in contrast to individual unclumped eggs which appeared to develop normally when placed in fresh liquid CeMM. Clumping was not observed on NGM plates, on CeMM plates, or when individual animals were cultured in liquid CeMM. These results suggest there are differences between CeMM and NGM grown animals.

Adaptation to growth in liquid CeMM

Animals in liquid CeMM grow more slowly than on NGM plates and appear to adapt to the medium. As shown in Figure 1, wildtype animals (Strain N2) grown on NGM plates reached a length of 1 mm in times consistent with those published [11]. Wildtype N2 animals that had been maintained in liquid CeMM for three years prior to this study took two to three times as long to reach 1 mm in length when grown in liquid CeMM (Fig. 1). Animals grown on 1.7% agar CeMM plates, reached a length of 1 mm in times not significantly different from those in liquid CeMM (Table 2), implying growth rate differences are the effect of CeMM composition but not the physical state. When animals were switched from NGM plates into liquid CeMM or from liquid CeMM to NGM plates, animals grew as predicted for the medium to which they were switched (not shown). Animals developed from eggs transferred into liquid CeMM from NGM plates had no gross phenotypic differences from animals exclusively grown in liquid CeMM. Animals developed from eggs transferred onto NGM plates from liquid CeMM displayed a moderate egg-laying defect (36%) and massive internal hatching (8%). These data suggest adaptation to growth in liquid CeMM occurs. Therefore we have provided the wildtype N2 grown in CeMM for three years with a new name under our registered lab designation, strain CC1. Using a wildtype strain maintained in axenic medium for several years in another laboratory (Strain LSJ1) we observed social feeding behavior when grown on NGM plates. In liquid CeMM LSJ1 took two days longer to reach 1 mm than either of the other wildtype strains (N2, CC1). Surprisingly, LSJ1 started laying eggs at approxi-

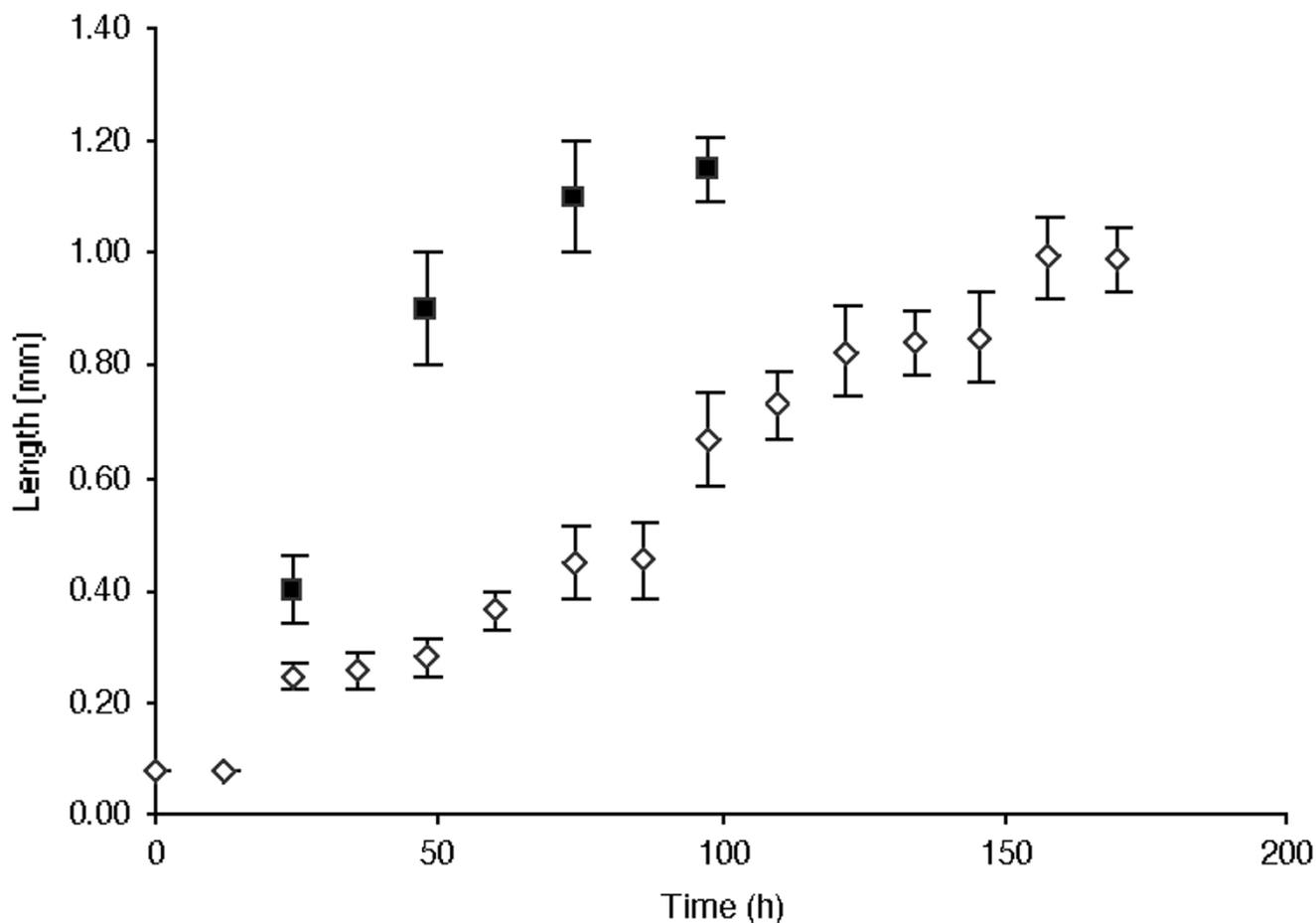


Figure 1

Growth curve at 20°C for animals grown on NGM plates (filled squares) and in liquid CeMM (open diamonds). Eggs were obtained by bleach treatment of animals grown for at least three years on NGM plates or in liquid CeMM. Approximately 50 eggs from NGM plate grown worms were placed on fresh NGM plates at $t = 0$. Approximately 50 eggs from liquid grown CeMM worms were placed in 50 μ l of fresh CeMM in a microfuge tube cap in a humidified chamber at $t = 0$. Length of ten animals was optically measured every twenty-four (NGM) or twelve (CeMM) hours, until adults were observed in consecutive timepoints.

mately the same time as strains N2 and CC1 despite being shorter. LSJ1 started laying eggs at 0.8 ± 0.04 mm as opposed to the > 1 mm classically associated with reaching the egg laying stage of adulthood [11]. These results suggest different adaptations to growth in axenic media are possible and that the life-cycle of CeMM grown animals is altered compared to that of animals on NGM plates.

Dynamic nature of CeMM cultures

CeMM composition changes as the result of animal growth in the medium and growth rates are consequently altered as the medium changes. When culturing at

22.5°C, liquid CeMM remains at pH 6 for the first week, increases to pH 7 after one week, increases to pH 8.5 after three weeks, and remains at pH 8.5 after six weeks, suggesting that animals condition CeMM. To evaluate growth in conditioned liquid CeMM, we used 75 cm² flasks to grow animals at 25°C for 4.5 weeks, at which point liquid CeMM had decreased in volume from 10 to 3.5 ml. Animals were killed by heating to 50°C for one hour. New animals added to this medium reached 1 mm one day sooner than animals grown in fresh CeMM subjected to 50°C for one hour (Table 2). Particulate material, in the form of shed cuticles and dead animals, accumulates in conditioned liquid CeMM. Particulate material has been

reported to increase the growth rate of the related species *C. briggsae* by providing physical stimulation [12]. To test if particulate material accounted for the increased growth rate we grew animals in conditioned, heat killed liquid CeMM subject to filtration through a 0.2 micron filter (Millipore). Animals reached a length of 1 mm three days later than animals grown in fresh liquid CeMM (Table 2), suggesting both that conditioned CeMM is nutritionally depleted or otherwise altered in a toxic fashion, and that the presence of accumulated particulate material improves the growth rate in liquid CeMM. We believe increased growth rate is the result of cannibalism of dead animals in liquid CeMM. Accordingly, we find addition of *E. coli* strain OP50 to fresh liquid CeMM results in growth rates not significantly different from animals grown in conditioned, unfiltered liquid CeMM (Table 2). Together these results also suggest that animals grown in fresh liquid CeMM are receiving suboptimal nutrients. It is important to note this is defined by growth rate which may or may not be the appropriate end point to measure [13]. These results suggest careful consideration should be given to using animals in fresh versus conditioned media for experimentation and that composition changes must be taken into account when comparing data from separate experiments.

CeMM can be stored, but repeated handling will decrease shelf life, presumably due to degradation of components because of photosensitivity or oxidation. Animals grown at 25°C displayed an unaltered growth rate in liquid CeMM that had been stored unused in the dark at 4°C for one year as compared to fresh liquid CeMM (Table 2). In contrast, animals displayed a variably decreased growth rate reaching 1 mm two to five days later than animals in fresh liquid CeMM when grown at 25°C in liquid CeMM stored in the dark at 4°C but subject to periodic removal for culturing animals over the course of a year (Table 2). We find storing and handling media in small aliquots minimizes concerns with media degradation.

Using standard protocols with CeMM grown animals

Most protocols developed for NGM grown animals work with CeMM grown animals. Standard protocols for settling worms or bleaching eggs to create age synchronous populations were easily performed with CeMM grown animals, as were protocols for preparation of dauers, dauer recovery, and freezing of strains [4,7]. Individual animals were manipulated using a pipettor and microscopic observation and optical measurement of animals were conducted with ease. Matings were not easily carried out but were possible on small CeMM plates in 24 well tissue culture dishes.

Much as with bleaching eggs from NGM grown animals, it is possible to overbleach CeMM grown animals and get

Table 2: Time animals take to reach 1 mm in length when CeMM is altered

Condition ^{L2}	Days to reach 1 mm at 25°C ³
Fresh liquid CeMM	5
Fresh CeMM plate	5
Fresh liquid CeMM heated to 50°C for 1 h	5
Liquid CeMM conditioned 4.5 weeks, heated to 50°C for 1 h	4
Liquid CeMM conditioned 4.5 weeks, heated to 50°C for 1 h, filtered	8
Fresh liquid CeMM with added <i>E. coli</i> strain OP50	4
Liquid CeMM stored 1 y in the dark @ 4°C	5
Liquid CeMM stored 1 y in the dark @ 4°C, subject to periodic removal from storage for use	7-10

¹ CeMM plates were 60 × 15 mm ² Liquid CeMM was 200 μ l in a 24 well tissue culture dish ³ All times represent the average length of ten animals from three independent runs

no viable eggs, underbleach CeMM grown animals and get eggs that develop within the incompletely dissolved parents, and it is possible to bleach just long enough to get eggs from which 98% or greater develop to adulthood. As with NGM grown animals the age, and thus strength, of the bleach solution and the density of worms being bleached appear to affect efficiency of bleaching CeMM grown animals.

On NGM plates dauer formation is determined by an integrated response to food, pheromone, and temperature [14]. While dauers may be in a developmental stasis, dauers are active living animals with an altered metabolism compared to adults [15,16]. Dauers generated at 27°C are often paler than dauers generated at 25°C [17] suggesting that dauers generated by various means are not the same. It therefore stands to reason that dauers generated by various techniques may not respond identically to changes in environment. Rather than study dauer formation and recovery for CeMM grown animals extensively, we simply assayed the small scale ability to generate and recover dauers. In three separate attempts, all of the viable progeny of four adults placed on CeMM plates at 28°C for one week were dauers and no dauer animals were noted on the plates after an additional week at 20°C.

The number of animals frozen directly impacts the number of animals recovered for both liquid CeMM grown animals and NGM plate grown animals. Since it is easier to generate larger volumes of worms using liquid CeMM than on a NGM plate it is possible to get larger

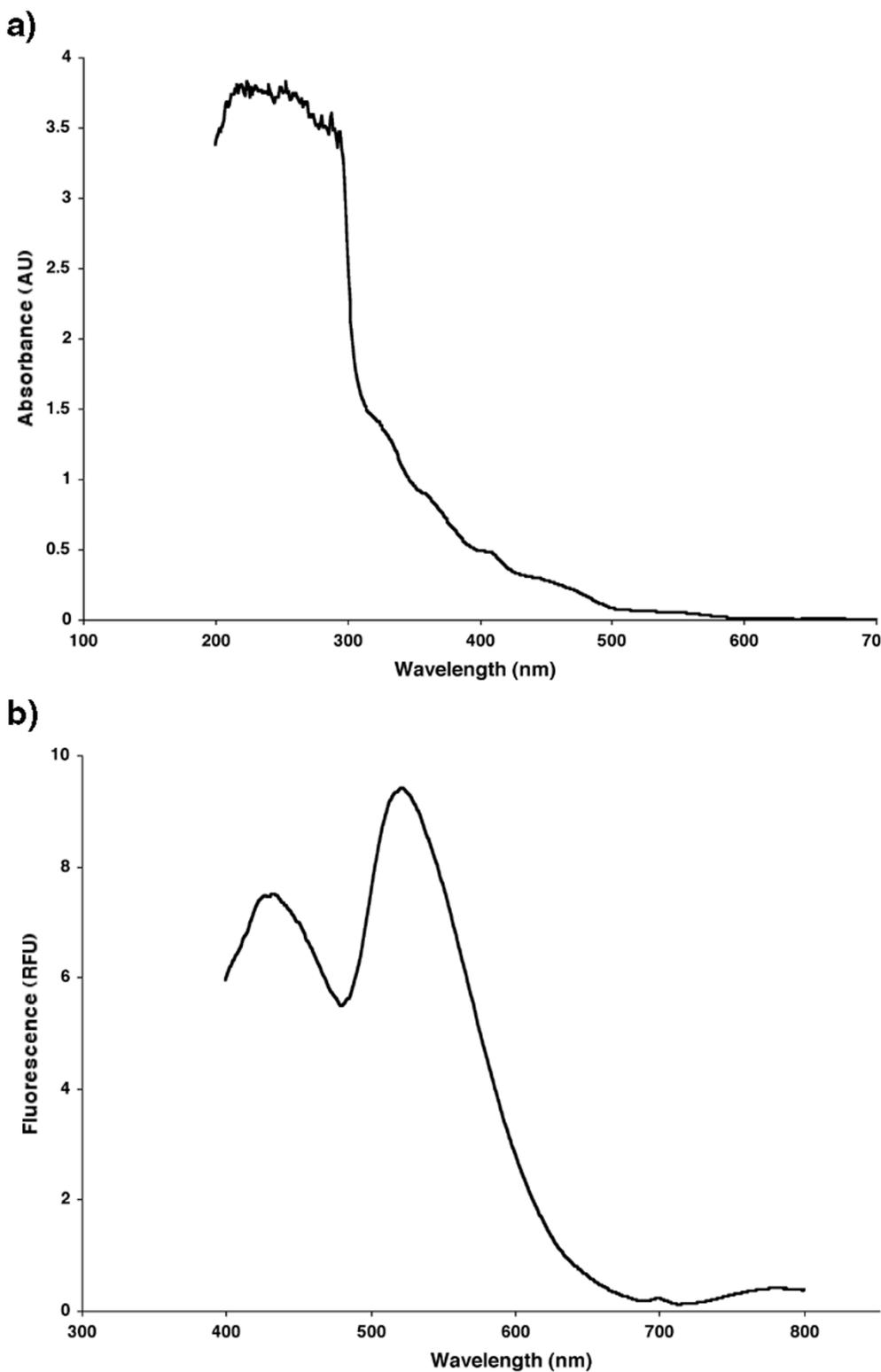


Figure 2

Absorbance and emission spectra for CeMM. a) Absorbance of CeMM is shown as a function of wavelength. Absorbance was measured for each nm increment between 200 and 1000 nm. No appreciable signal was noted between 700 and 1000 nm. b) Emissions of CeMM as excited at 350 nm. Emissions were measured for each nm increment between 400 and 800 nm.

numbers of animals surviving freezing. Mixed stage animals grown in liquid CeMM can be directly mixed 1:1 with freezing medium [4,7] and slowly frozen to -80°C . Compared to typical starved L1 freezes prepared from NGM grown animals, larger numbers of animals are viable after three years. We have not attempted to quantitate viability nor compare viability between culture conditions. We also have not attempted to generate starved L1 freezes from liquid CeMM grown animals even though starved L1s have been reported to freeze better than mixed stage NGM preparations [4,7]. The ability to recover large numbers of viable animals from liquid CeMM cultures may indicate CeMM grown animals are partially starved, are somehow otherwise prone to survive freezing better than NGM grown animals, or may simply reflect the ease with which large numbers of animals can be generated in liquid CeMM.

CeMM has a very broad absorbance spectra ranging from below 200 to just below 600 nm (Fig 2a). CeMM has two major emission peaks. When excited at 350 nm these peaks are centered at 434 and 522 nm (Fig 2b). This is a widely used range for a number of applications therefore these emissions may present a difficulty for some applications. Due to CeMM fluorescence GFP was difficult to observe in transgenic animals containing GFP in nerve and muscle when a dissecting microscope was used. (Strains MU1085 (*bwl-2(flp-1::gfp; rol-6(su1006))*) and PJ707 (*jls01(myo-3::GFP; rol-6(su1006))*)). When viewed with a compound microscope we experienced no difficulty in observing GFP and little problem with background fluorescence from CeMM. We believe the difference in GFP visualization on our microscopes reflects the relative depth of liquid CeMM in which worms were observed. However, width of field and slight differences in the filter sets used are other potential explanations.

The only procedure we had difficulty carrying out with CeMM grown animals was mating. Consistent with the *a priori* prediction that males would not mate well in liquid, we observed successful matings on CeMM plates but not in liquid CeMM, both in 24 well tissue culture dishes (Strains CB1489 (*him-8(e1489)* IV) and PJ1046 (*cha-1(p1182^{ts})* IV; *ccIs55(unc-54::lacZ)* V)). Successful mating was noted on one of three attempts on a CeMM plate made in a 24 well tissue culture dish and not at all on three attempts on a CeMM plate made in a 60×15 mm or 35×15 mm petri dish. We presume matings were unsuccessful most of the time because the animals move throughout the plate rather than remaining on the surface. As such it was possible to observe cross progeny but not determine mating efficiency. It is possible that male mating ability is reduced when animals are grown using CeMM, however we have no data on this. These results

demonstrate that although there may be differences between NGM and CeMM grown animals, knowledge of NGM grown animals can be applied to CeMM grown animals.

Conclusions

We have presented technical aspects of our initial work with animals grown in liquid CeMM. These animals are clearly different from those grown on NGM plates. Those wishing to use CeMM should be aware that, as with all media, there are caveats associated with use of CeMM and potential pitfalls of applying what is known of animals on NGM plates to animals grown using CeMM.

Authors' contributions

All authors participated in study conception, manuscript preparation, the testing of laboratory hardware, and the testing of procedures with animals grown in CeMM. NJS performed the growth rate, media switching, and effects of conditioned medium experiments. EK performed the experiments to assess pH changes of the medium.

Additional material

Additional File 1

Chemically Defined C. elegans Medium Preparation. This file is a protocol adapted from Lu & Goetsch (1993) for the preparation of CeMM.

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Acknowledgements

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