Several micronutrients in the rotifer *Brachionus* sp. may not fulfil the nutritional requirements of marine fish larvae

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Abstract

The current best practice intensive culture of larval Atlantic cod includes feeding rotifers from onset of exogenous feeding until 25–30 days after hatching. These larvae grow considerably slower and develop higher frequencies of deformities than larvae reared in semi-extensive systems, using copepods as feed. The present study compares the micronutrient concentrations in rotifers with those of copepods, with the aim of identifying nutrients that may be limiting for normal growth and development of cod larvae. An additional criterion used is the nutrient requirements given for fish in general, by NRC (1993), as nutrient requirements of cod remains to be determined. Rotifers were fed on four different diets, consisting of baker's yeast with cod liver oil (3.3 : 1 dry weight (DW)/v), baker's yeast with Algamac 2000^{TM} (3.5 : 1 DW), baker's yeast with live algae *Chlorella* (4.1 : 1 DW), and Culture Selco 3000^{TM} (CS). CS was a complete commercial diet for rotifers while the other diets are considered as based on raw ingredients. Compared with copepod nutrient levels, rotifers grown on yeast-based diets supplemented with either cod liver oil, Algamac 2000 or *Chlorella* were apparently sufficient for covering the requirements in cod larvae for all the B-vitamins, except thiamine. Rotifers cultured on the CS diet also had sufficient amounts of thiamine. Of the minerals, only calcium and magnesium were sufficient, using this criterion while iron was on the borderline. However, with reference to the requirements given for larger fish (NRC 1993), only thiamine, vitamin A, manganese, selenium and perhaps copper, appear too low in the rotifers cultured without extra micronutrient supplementation. The other nutrients were present at levels intermediate between copepod and fish requirement levels. This study suggests that it is necessary to develop enrichment techniques to produce rotifers with sufficient amounts of all micronutrients. Such techniques will also be important tools for determining which nutrients are present at levels below the actual requirements in cod larvae.

KEY WORDS: cod larvae, minerals, requirements, rotifers, rotifer diets.

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Introduction

Cod larvae reared in semi-extensive systems and fed on natural marine zooplankton, generally show very high specific growth rates (13–25% per day, Otterlei *et al*. 1999; Finn *et al*. 2002). The growth rates of cod larvae cultured in intensive systems are normally lower than this (approximately 10% per day, MacQueen Leifson 2003). Further, batches of juveniles arising from semi-extensive larvae are more robust and have lower rates of deformities than those from intensively reared larvae (Imsland *et al*. 2006). There are many possible reasons for these differences, where environmental factors, bacterial and viral activity and nutrition may play significant roles. It is important to optimize all these factors to obtain good culture practises, with larval growth rates and juvenile quality similar to that observed for semi-extensively reared larvae.

The aim of the present study was to characterize the micronutrient levels in rotifers reared on diets based on raw ingredients which are used in the industry (not supplemented with micronutrients), and compare them with nutrient levels in copepods and the requirements given for juvenile and adult cold-water fish (NRC 1993). A complete culture diet for rotifers, Culture Selco 3000, was used for comparison and

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to give an indication of the nutrient levels actually present in hatchery reared rotifers fed commercial diets. In the same experiment, protein and lipid levels and amino acid, fatty acid and lipid class composition were also measured (Srivastava *et al*. 2006; Srivastava *et al*. unpublished). Altogether, this will identify which nutrients that are given potentially at levels below the requirements of fish larvae fed rotifers. Identification of possibly limiting nutrients in rotifers may give an explanation of the lower growth and vitality of fish larvae reared in intensive, compared with semi-extensive, systems.

Materials and methods

Culture of Brachionus sp.

The rotifers used in the present study were imported to Norway from Europe in 1998 by Stolt Sea Farm, Øye, and have been held in culture at this hatchery ever since. It is a member of the genus *Brachinus*, but the exact species has not yet been determined. It is smaller than *Brachinus plicatilis* and can be cultured in full seawater (Baer 2007). Presently, these rotifers are being distributed to several hatcheries in Norway, which use them as live feed for their cod larvae.

The rotifers were cultured at Stolt Sea Farm AS, Øyestranda, Norway, in 1000 L fibre glass tanks with conical bottom, in two different experiments. In experiment 1, rotifers were fed with four different diets: bakers yeast with cod liver oil (3.3 : 1 dry weight (DW)/volume, Peter Møller, Lysaker, Norway; DYO); bakers yeast with Algamac 2000^{TM} (3.5 : 1 DW, Aquafauna Bio-marine, Inc., Hawthorne, California, USA; DYA) which, according to the supplier is a pure, spray dried, hetrotrophic algae, *Schizochytrium* sp.; bakers yeast with live algae *Chlorella* (4.1 : 1 DW; Pacific Trading Co. Ltd., Tokyo, Japan; DYC) and Culture Selco 3000^{TM} (INVE, Baasrode, Belgium; DCS). In experiment 2, rotifers were cultured with baker's yeast–oil–live *Chlorella* (65 : 25 : 15 DW). The oil was a synthetical fish oil, EPAX 5010, from Pronova AS, Sandefjord, Norway, having EPA and DHA levels of 10% and 50% of fatty acids, respectively.

To compensate for the lack of replicate culture tanks, both experiments were repeated three times. In experiment 1, rotifers grown on the standard Øye diet (Baker's yeast–cod liver oil (10 : 1, weight/volume) with vitamin supplement and live algae *Isochrysis*), was used as inoculums for each new growth cycle. These rotifers had been held in culture in the hatchery and fed the Øye diet for several years. All data, except those on macromineral composition of rotifers are derived from experiment 1. In experiment 2, the inoculums were from a rotifer stock culture grown with the baker's yeast–Pronova oil–*Chlorella* (65 : 25 : 15 DW) diet, i.e. the rotifers in the inoculums had been fed the experimental diet on a long-term basis. The data in Table 1 on macromineral composition of rotifers are derived from experiment 2. Initial inoculums density was 350–400 rotifers per mL.

The rotifers were cultured in a batch system and no water exchange was performed during the 4-day culture cycles. Particles were removed daily by sedimentation. The rotifers were fed 50–150 g diet dry matter per day, varying with the rotifer density and appetite. The rotifers were not fed above the satiation level to avoid water quality deterioration. Water temperature varied between 23 °C and 25 °C, salinity between 30 and 32 ppt and pH between 7.05 and 7.71 during the culture period, measured with Metrohm 632 pH-Meter, Switzerland. Oxygen was measured using a DO meter, model 550A from Yellow Springs Instruments (YSI), USA.

All rotifers were harvested after 4 days. At the time of harvest, the rotifer cultures were filtered (65 μm, mesh size), and rinsed in tap water. The concentrated rotifers were packed and stored in liquid nitrogen for vitamin analyses and at −20 °C for mineral analyses before being transported to NIFES, Bergen, Norway, for analysis. At NIFES, vitamin samples were kept at −80 °C and mineral samples at −20 °C, until analysed. Samples were also collected from all diet ingredients.

Collection of copepods

As reference data for copepods, previously collected and analysed samples from Haapollen were used. Haapollen is an enclosed seawater lagoon at a halibut fry production plant in Western Norway (Stolt Sea Farm, Aga). These natural copepods were used as feed to cultured halibut larvae and resulted in high-quality juveniles with good growth and low incidence of deformities. The sampling period was from May 3 to June 24 in 1994 and from April 5 to June 27 in 1995 and covered most of the period when the halibut were fed copepods. The salinity of the lagoon water was 28–30 g L^{-1} during

Table 1 Rotifers – macrominerals (mg kg^{-1} dry weight) in rotifers grown on a diet of yeast, Pronova oil and *Chlorella* (3 : 1 : 1 on a dry matter basis), compared with copepod levels and the requirements in cold-water fish given by NRC (1993)

	Rotifers	Copepods	NRC
Phosphorus	9400 ± 690	12400–15010	4500–6000
Calcium	1880 ± 220	1070–2370	Not determined
Magnesium	4840 ± 450	2350–3140	400–600

both seasons while the water temperature increased from about 7.5–13 °C and 4.5–14 °C, in 1994 and 1995, respectively. The plankton samples consisted of the 180–460 μm fraction (UNIK filter systems) of the natural lagoon plankton (Berg 1997), mainly copepodites of *Temora longicornis* (Fyhn *et al*. 1995). To obtain DW for individual plankters, known volumes (0.5 L) of counted samples of the plankton fraction were filtered through a Millipore suction filter system (40 μm). Micronutrient composition was measured in pooled samples (approximately 1 g wet weight) collected from the Millipore filter system, frozen on dry ice in Nunc cryotubes, and stored at −80 °C until the day of analysis.

Analytical methods

Dry weight was determined gravimetrically after drying the samples at 105 °C over night. Vitamins A and E were determined by normal phase HPLC after saponification and extraction of the sample by hexane (Lie *et al*. 1994; Nöll 1996; Moren *et al*. 2004a). Ascorbic acid was analysed by HPLC with amperiometric electrochemical detector as described in Mæland & Waagbø (1998). Culture Selco 3000 and the rotifers fed on this diet, were treated with acid phosphatase after extraction for the removal of phosphate from ascorbate-2-phosphate. The B-vitamins, except for thiamine and pyridoxine, were analysed using semi-automated microbiological assays as described in Mæland *et al*. (2000). Thiamine analysis was carried out by HPLC according to CEN (Comitè Europèen de Normalisation), TC 275 WI 002750053 N134 (2002a)) and pyridoxine, also an HPLC method, according to CEN (Comitè Europèen de Normalisation) TC275 WI 00275131 (2002b) and Albrektsen *et al*. (1993) modified after AOAC (Association of Official Analytical Chemists) (1990). Extraction and analyses of the different carotenoids from the diets was conducted as described by Torstensen *et al*. (2004). For trace element analyses, freeze-dried samples of diet ingredients and rotifers were wet digested by use of a microwave technique in nitric acid with 30% hydrogen peroxide (Julshamn *et al*. 2000). The samples were then analysed for Fe, Mn, Cu, Zn and Se according to Julshamn *et al*. (2004) and for P, according to Liaseth *et al*. (2003). Mg and Ca were analysed according to Julshamn *et al*. (1998), while iodine was analysed according to Julshamn *et al*. (2001). Ammonium was analysed using a salicylate-hypochlorite method adapted for determination of small amounts ammonium in seawater (Bower & Holm-Hansen 1980). The samples were added a salicylate–catalyst solution and an alkaline hypochlorite solution to give a purple-colour reaction. Absorbencies were measured in a Shimadzu UV-160 spectrophotometer at 650 nm. Standards were prepared from ammonium chloride.

Samplings for total number of bacteria were performed during the time of inoculum (0 day) and at the end of culture cycle (4th day). Total number of bacteria were counted with the help of a fluorescent microscope after staining with DAPI (4,6-diamindino-2-phenylindiole) a modified method of Porter and Feig (1980). In brief, culture water samples preserved with 4% formalin, were homogenized with sonication for few seconds and then applied on the 0.2 μm polycarbonate filter supported with a GF/C filter. After low-pressure suction of water, the filter was stained with DAPI. After 30 min of staining, the filter was mounted on a slide in paraffin and examined under a fluorescent microscope.

The number of rotifers along with the egg rotifer ratio was counted daily under a microscope after random sampling of the water from the culture tanks (100 mL) followed by 1 mL sub-sampling from the collected sample. The sub-sample was then fixed and stained with lugol and the stained rotifers were counted under the microscope.

Statistics

The calculation of un-ionized ammonia was based on Fivelstad (1988). The software Statistica (ver. 7, Statsoft Inc. Tulsa, OK) was used for the statistical analyses. Possible differences between treatments were analysed by one-way ANOVA, followed by Scheffes *post hoc* test, when Levene's test showed that the variances were homogenous. Change in concentration or rotifer performance with time was analysed by ANOVA for repeated measurements. Means with non-homogenous variances were analysed by Kruskal–Wallis ANOVA and Median test. Differences were considered significant at $P < 0.05$.

Results

Culture parameters

There were no differences in growth or egg ratio between the dietary treatments in experiment 1, but the variation was high, so possible effects of the diets on these parameters may have been masked by random variation in the culture conditions. The number of rotifers per mL increased from 351 ± 16 on day 0 to 718 ± 120 after 4 days of culture in experiment 1 and from 374 ± 75 to 1051 ± 89 in experiment 2. In experiment 1, the egg ratio varied between 0.26 ± 0.04 eggs per rotifer on day 0 and 0.32 ± 0.01 on day 3, with no significant differences neither between dietary

treatments, nor between days. Egg ratio in experiment 2 varied between 0.25 ± 0.07 and 0.40 ± 0.06 on days 4 and 2, respectively, and egg ratio on day 2 was significantly higher than on days 1 and 4 ($P < 0.02$). The egg ratio on day 3 was intermediate. Oxygen concentration just before feeding decreased from 6.6 ± 0.2 to 5.5 ± 0.4 mg L^{-1} during the repetitive feedings in the first experiment and from 7.48 ± 0.23 to 4.54 ± 0.89 in the second experiment. After feeding, there was a drop in oxygen concentration which was only monitored in the second experiment. Here, oxygen concentration dropped to between 2 and 5 mg L^{-1}, 1 h after feeding and then increased again. In experiment 1, total ammonia increased significantly from 21 ± 13 μmol L^{-1} in the inoculums to 70 ± 27, 417 ± 105, 586 ± 85 and 420 ± 66 μmol L^{-1} on day 4, in the YO, YA, YC and CS treatments, respectively ($P < 0.02$). Ammonia concentration was significantly lower in the YO treatment than in the other groups ($P < 0.002$). The range of un-ionized NH_3 on day 4 of the experiment was 0.4–13.6 μmol L^{-1}, corresponding to 0.006–0.23 mg L^{-1}. Total bacterial count in experiment 1 did not differ between the dietary treatments. When all groups were pooled, there was a significant increase in bacterial count from 2.6 ± 1.3 10^5 in the inoculums to 15.8 ± 7.4 10^5 on day 4 of the experiment ($P = 10^{-4}$).

Vitamins

Rotifers fed the YO, YA and YC diets, which were not supplemented with vitamin C, had vitamin C levels between 100 and 200 mg kg^{-1} DW, which was a significant reduction from 516 mg kg^{-1} in the inoculums ($P < 0.002$, Table 2). Rotifers fed the CS diet had no change in vitamin C concentration, compared with the inoculums and higher levels of vitamin C than the other groups ($P < 0.02$, Table 2). This corresponds well with diet (Table 2) and ingredient (Table 3) concentrations of vitamin C, where the diets based on raw ingredients, only (YO, YA and YC), contained less than 25 mg kg^{-1} vitamin C and the CS diet, which is a complete diet for rotifers, contained more than 1000 mg kg^{-1} vitamin C.

Yeast appears to be a good source of B vitamins, in relation to the criteria used in the present study to indicate the requirements of cod larvae. The concentrations of B vitamins, with exception of thiamine, were at or above the concentrations found in copepods and considerably higher than the recommendations given by NRC (1993) for larger fish in all rotifer treatment groups. Thiamine concentration in rotifers grown on YO and YC decreased significantly ($P < 0.04$), compared with the inoculums and was 3–6 times lower than the lowest concentrations measured in copepods (Table 2). Rotifers fed the YA diet had a high average concentration of thiamine, but the within treatment variation was large, so that the lower replicates were similar to the YO and YC rotifers. The dietary thiamine is largely derived from the yeast in the both the YO and YA treatments while in the YC treatment, *Chlorella* also contributes with thiamine (Table 3). CS had a four-fold higher thiamine concentration than yeast and *Chlorella* and 26-fold higher concentration

Table 2 Mixed diets and rotifers – vitamin levels (mg kg^{-1} dry weight) in rotifers and the different diets used in experiment 1, compared with copepod levels and the requirements in cold-water fish given by NRC (1993)

	Diet				Rotifers						
	YO	YA	YC	CS	Start	YO	YA	YC	CS	Copepods	NRC
Vitamin C	0.31	9.9	22	1135	516 ± 128	181 ± 105^{b}*	190 ± 160^{b}*	117 ± 68^{b}*	576 ± 84^{a}	500	50
Riboflavin	58	64	72	24	20 ± 3	43 ± 3^{a}*	22 ± 0^{b}	44 ± 1^{a}*	23 ± 1^{b}	14–27	4–7
Thiamine (B1)	32	34	43	192	70 ± 9	2.0 ± 1.3*	57 ± 40	4.2 ± 2.3*	125 ± 87	13–23	1.00
Folic acid	8.8	9.3	14.0	18	5.4 ± 2.8	5.7 ± 0.5	5.1 ± 0.7	4.0 ± 0.9	5.6 ± 0.8	3–5	1
Pyridoxine (B6)	28	29	37	16	49 ± 10	25 ± 8^{b}*	20 ± 3^{b}*	23 ± 4^{b}*	53 ± 2^{a}	2–6	3–6
Biotin	0.48	0.99	1.09	0.56	1.4 ± 0.2	1.5 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	1.8 ± 0.1	0.6–0.9	0.15–1
Cobalamin (B12)	0.10	0.90	4.61	7.2	26 ± 2	26 ± 9^{b}	23 ± 8^{b}	43 ± 8^{ab}	61 ± 9^{a}*	1–2	0.01
Niacin	174	182	223	364	202 ± 6	191 ± 14^{b}	249 ± 40^{ab}	192 ± 12^{b}	267 ± 6^{a}*	100–150	10–28
Vitamin E	677	1.38	45	4233	469 ± 126	294 ± 37^{ab}	91 ± 86^{b}*	85 ± 74^{b}*	889 ± 547^{a}	110	50
Carotenoids	0.12	0.54	1.47	1.32	23 ± 2	4.3 ± 0.6^{b}	14 ± 2^{a}*	14 ± 1^{a}*	15 ± 3^{a}*	630–750†	
Vitamin A	15.4	0.13	1.16	205	3.1 ± 0.5	0.00^{b}*	0.00^{b}*	0.00^{b}*	9.5 ± 1.8^{a}*	0	2.4§

YO, Yeast and oil; YA, yeast and Algamac 2000; YC, Yeast and *Chlorella*; CS, Culture Selco 3000. (Mean ± SD, $n = 3$).
Different superscript alphabets indicate significant differences between dietary treatments.
* Significant change in nutrient concentration during the feeding experiment, $P < 0.05$.
† van der Meeren (2003).
§ Moren *et al.* (2004b).

Table 3 Feed ingredients – vitamin levels (mg kg^{-1} dry weight) in the feed ingredients used for culture of rotifers in experiments 1 and 2

	Yeast	Algamac 2000	*Chlorella*	Culture Selco	Cod liver oil
Vitamin C	0.40	43	108	1135	
Riboflavin	75	25	60	24	
Thiamine (B1)	42	7.4	48	192	
Folic acid	11.5	1.7	24	18	
Pyridoxine (B6)	36	4.9	41	16	
Biotin	0.62	2.28	3.0	0.56	
Cobalamin (B12)	0.13	3.61	23	7.2	
Niacin	227	26	206	364	
Vitamin E	0.77	3.5	225	4233	2908
Carotenoids	0.15	1.90	6.9	1.32	0.02
Vitamin A	0.00	0.59	5.9	205	66

than Algamac 2000. This was partly mirrored in the significantly higher thiamine concentration in the CS rotifers compared with the YO and YC groups ($P < 0.02$, Table 2).

Cod liver oil and Culture Selco 3000 contained 2900 and 4200 mg kg^{-1} vitamin E, respectively, the level in *Chlorella* was 225 mg kg^{-1} and in yeast and Algamac 2000, less than 10 mg kg^{-1}. The dietary concentrations of vitamin E were mirrored in the rotifers, where the CS rotifers contained significantly more vitamin E than the YA and YC rotifers ($P < 0.05$), and the YO rotifers were intermediate (Table 2). The carotenoid concentrations (astaxanthin + cantaxanthin) were generally very low in rotifers, compared with copepods, rotifers given the YO diet having significantly lower levels of carotenoids than the other groups ($P < 0.025$, Table 2). The dietary concentrations of carotenoids were less than 2 mg kg^{-1}. The concentrations of vitamin A in yeast, Algamac 2000 and *Chlorella* were less than 6 mg kg^{-1}, cod liver oil contained 66 mg kg^{-1} and Culture Selco 3000 contained 205 mg kg^{-1}. Vitamin A was not detected in YO, YA and YC rotifers, while the CS rotifers contained 9.5 mg kg^{-1}, which is approximately four-fold higher than the apparent optimum level of vitamin A for Atlantic halibut juveniles (Table 2, Moren *et al.* 2004b).

Minerals

Of the macrominerals, phosphorus was lower in rotifers grown on a mixture of yeast, *Chlorella* and Pronova oil, in experiment 2, than in copepods, while calcium and magnesium were higher in rotifers than in copepods (Table 1).

There were no differences in iodine concentration between rotifers on the different dietary treatments and no change in iodine concentration during the culture period (Table 4). All the dietary ingredients contained less than 1 mg kg^{-1} iodine (Table 5). The iodine concentration in rotifers was 6- to 116-fold lower than the concentrations measured in copepods (50–350 mg kg^{-1}), but slightly higher than the requirements give by NRC (1993) for larger fish (Table 4). Manganese in rotifers varied significantly between the dietary treatments, but the concentration differences were small (3.9–5.1 mg kg^{-1}, Table 4). *Chlorella* contained quite high levels of manganese (Table 5), but this was only slightly reflected in the rotifer concentrations (Table 4). The levels of manganese in rotifers were below both copepod levels and the recommendations given by NRC (1993). The diets based on raw ingredients, only (YO, YA, YC) contained less than 2 mg kg^{-1} copper and rotifers fed these diets contained approximately 3 mg kg^{-1} copper, similar to the requirements for fish (NRC 1993). The copper concentration in rotifers fed the CS diet, which was probably supplemented with copper (Table 5), was significantly higher than in rotifers from the other treatments (8.1 mg kg^{-1}, $P < 0.006$), but still below the concentrations found in copepods. The concentration of zinc in rotifers did not differ between the dietary treatments and

Table 4 Mixed diets and rotifers – microminerals (mg kg^{-1} dry weight) in rotifers and the different diets used in experiment 1, compared with copepod levels and the requirements in cold-water fish given by NRC (1993)

	Diets				Rotifers						
	YO	YA	YC	CS	Start	YO	YA	YC	CS	Copepods	NRC
Iodine	0.023	0.058	0.042	0.158	3.7 ± 0.5	5.8 ± 3.8	3.2 ± 3.4	7.9 ± 2.3	3.0 ± 0.5	50–350	0.6–1.1
Manganese	6.0	6.8	14.9	6.1	4.4 ± 0.2	3.9 ± 0.3^{b}	4.4 ± 0.2^{ab}	5.1 ± 0.4^{a}	4.3 ± 0.2^{b}	8–25	13
Copper	1.0	1.9	1.9	50	3.4 ± 0.5	2.7 ± 0.4^{b}	3.1 ± 0.4^{b}	3.1 ± 0.5^{b}	8.1 ± 2.2^{a}	12–38	3–5
Zinc	98	101	105	120	49 ± 3	62 ± 8	63 ± 5	64 ± 5	63 ± 3*	340–570	20–30
Selenium	0.02	0.04	0.03	0.08	0.08 ± 0.00	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.09 ± 0.02	3–5	0.25–0.3
Iron	106	114	156	94	123 ± 26	88 ± 17^{ab}	84 ± 14^{ab}	114 ± 23^{a}	57 ± 7^{b}	85–371	30–150

Mean ± SD, $n = 3$.
YO, Yeast and oil; YA, yeast and Algamac 2000; YC, Yeast and *Chlorella*; CS, Culture Selco 3000.
Different superscript alphabets indicate significant differences.
*Significant change in nutrient concentration during the experiment, $P < 0.05$.

Table 5 Feed ingredients – trace elements (mg kg^{-1} dry weight) in the feed ingredients used for the culture of rotifers

	Yeast	Algamac	*Chlorella*	Culture Selco
Iodine	0.030	0.157	0.092	0.158
Manganese	7.85	3.15	43.9	6.11
Copper	1.33	4.00	3.99	50
Zinc	127	7.53	10.3	120
Selenium	0.03	0.06	0.01	0.08
Iron	138	28.4	232	94

was higher than the requirements of fish but considerably lower than the concentrations in copepods. Yeast and Culture Selco 3000 have approximately 120 mg kg^{-1} zinc, while Algamac 2000 and *Chlorella* are poor sources of zinc. Selenium in rotifers was similar in all groups and considerably lower than both fish requirements (NRC 1993) and copepod levels. The dietary ingredients all contained less than 0.1 mg kg^{-1} selenium, while the requirement in fish is 0.25–0.3 mg kg^{-1}. The concentration of iron in rotifers reflected the concentration in the diets, being higher in rotifers fed the YC diet than in those fed the CS diet ($P < 0.02$). The two other groups had intermediate levels of iron. Yeast and *Chlorella* were good sources, while Algamac 2000 and probably fish oil (not analysed), were poor sources of iron (Table 5). Culture Selco 3000 gave rotifers with iron levels below the levels in copepods and in the lower range of requirements given for fish (Table 4, NRC 1993). The other diets gave iron levels in the lower range of those found in copepods.

Discussion

Using copepod nutrient levels as the criterion, the present study shows that of the analysed vitamins, only the B-vitamins, except thiamine, appear to be sufficient for covering the requirements in cod larvae, in rotifers grown on yeast-based diets supplemented with either cod liver oil, Algamac 2000 or *Chlorella*. Of the minerals, only calcium, magnesium and perhaps iron were sufficient, using this criterion. However, according to the requirements given for larger fish (NRC 1993), only thiamine, vitamin A, manganese, selenium and perhaps copper, are too low in the rotifers cultured without extra micronutrient supplementation. The data on NRC recommendations are given as the range of requirements in all species listed, as there are no requirement data for cod.

Because of the difficulties in performing requirement studies with live feed and the limited success of development of formulated diets that can be used in such studies (Kvåle *et al.* 2007), the exact nutrient requirements of marine fish larvae are not known. Although fish larvae in the oceans may feed on algae and different plankton organisms, copepods are the main feed for wild fish larvae, and in the present study it is assumed that this group of feed organisms will cover the larval requirements. Copepods are very rich in nutrients and it is likely that fish larvae have adapted to ingesting and digesting these prey organisms during evolution and therefore accordingly have high requirements. However, it is also possible that the actual requirements of the larvae are less or even much lower than what they get through their natural feed. Until feeds that allow manipulation and proper control of the nutrient composition are available, these hypotheses cannot be tested. Therefore, the nutrient requirements of juvenile and adult fish given by NRC (1993) were used as an additional criterion in this study. However, it is generally believed that small fish have higher nutrient requirements than adults and using adult values may underestimate the requirements. Fish larvae may have specific growth rates in the range of 30% per day (Otterlei *et al.* 1999), which suggest that they need a nutritious feed. Thus, the criteria used in the present study are not absolute criteria of fish larval requirements, but merely give an indication of rotifer adequacy.

Not only the levels of micronutrients, but also the bioavailability is important for evaluation of the nutritional value of different feeds for marine fish larvae. This is especially true for minerals, where the molecular form of the mineral, the solubility of this form in the feed matrix and gut lumen and binding within tissues with different digestibility will affect the fraction of the mineral which is absorbed (Andersen *et al.* 1997; Roy & Lall 2003). An additional factor concerning availability of nutrients in marine fish larvae is that their digestive tract develops from a larval type with low digestive capacities at first-feeding, to an adult type with fully developed digestion in juveniles (Kjørsvik *et al.* 1991; Pedersen & Falk-Pedersen 1992; Cahu & Zambonino Infante 2001; Kjørsvik *et al.* 2004; Kvåle *et al.* 2007; Rønnestad *et al.* in press). This may also affect the availability of micronutrients. However, availability measurements within complex matrices, such as whole feed organisms, is complicated and beyond the scope of the present study.

Another aspect which must be taken into account is that fish, and probably fish larvae, can utilize waterborne minerals (Watanabe *et al.* 1997), which are present at much higher concentrations in seawater than in freshwater and may vary in concentration between inshore waters and oceans and between up welling and less nutrient rich areas. The dietary requirements for trace elements in fish larvae are therefore to some extent dependent on the water quality. Further it appears that the mineral concentrations in copepods cultured

in fertilized lagoons are higher than in those filtered from the open sea (Hamre *et al.*, unpublished), indicating that the copepod mineral concentrations reported in the present study are in the high range of those encountered by cod larvae in the wild.

The culture conditions

The rotifers were cultured in a batch system which means that no water exchange was performed during the 4-day culture cycles. However, particles were removed daily by sedimentation. Such culture conditions lead to an increase of metabolites and increased microbiological activity. Still, such culture conditions give very stable production in rotifers as long as levels for ammonia, oxygen and pH are kept within normal levels (J. Stoss, unpublished observations). Both pH and oxygen concentration stayed well within a range which assures normal growth and reproduction (J. Stoss, unpublished observations). The $NH_3 : NH_4^+$ ratio is influenced by the temperature and the pH of the water. High levels of unionized ammonia are toxic for rotifers, but according to Dhert (1996), rearing conditions with NH_3 concentrations below 1 mg L^{-1} appear to be safe. In the present study, free ammonia stayed below 0.3 mg L^{-1}.

Bacteria are always associated with mass production of rotifers and may cause unexpected mortality or suppressed growth to rotifers. The billions of rotifers and their accompanying food inevitably create a high load of organic material, which allows the bacteria to grow fast. Usually, the aerobic bacteria population range from 10^4 to 10^7 CFU mL^{-1} in culture water (Miyakawa & Muroga 1988; Nicolas *et al.* 1989; Tanasomwang & Muroga 1990; Skjermo & Vadstein 1993). The bacterial load in the rotifer cultures in this study ($15.8 \pm 7.4\ 10^5$ CFU mL^{-1}) was thus intermediate compared with the normal range.

Vitamins

The vitamin C levels of rotifers grown on the YO, YA and YC diets declined compared with the levels in the inoculums, but were much higher than the vitamin C levels of the diets. As the rotifers were only cultured for 4 days on the experimental diets, it is possible that the vitamin C levels were still on their way down at the time of sampling and that additional culture cycles, using the previous culture as inoculums, would have given vitamin C levels that would be below the requirements in fish (NRC 1993). Vitamin C is one of the few micronutrients where requirements in fish larvae have been addressed (Merchie *et al.* 1997; Kolkovski *et al.* 2000). Merchie *et al.* (1997) found that 20 mg kg^{-1} ascorbic acid was sufficient for normal growth and survival of post-larval turbot and sea bass, when using formulated diets. The vitamin C concentrations found in live feed prior to enrichment, were sufficient for several fish and shrimp species. However, boosting the live feed organisms with vitamin C, up to 2500 mg kg^{-1}, improved stress resistance. Kolkovski *et al.* (2000) found improved stress resistance and survival of freshwater walleye fed high levels of long-chain n-3 PUFA by *Artemia* boosted with vitamin C from 300 to approximately 1500 mg kg^{-1} DW. The vitamin C content of rotifers fed on the CS diet would therefore have sufficient vitamin C to sustain growth and survival of marine fish larvae, but higher levels may be necessary to enhance stress resistance. Diets based on the raw ingredients used in this study should be supplemented with vitamin C to meet the larval requirements.

Except for thiamine, the B vitamin levels were higher in all rotifer groups than in copepods and the levels can therefore be assumed to be sufficient for cod larvae. Yeast is a good source of the B vitamins and rotifers cultured without yeast should be checked for these vitamins. Thiamine was low in rotifers cultured on the YO and YC diets but sufficient in rotifers fed the CS diet which is probably fortified with thiamine. Rotifers fed the YA diet had a high mean level of thiamine but the variation was large and the lower replicates were similar in thiamine to rotifers fed the YO and YC diets. The cause of this variation is not known. Thiamine level in *Artemia* is similar or slightly higher than the lowest levels found in rotifers in the present study (Mæland *et al.* 2000), and supplementing *Artemia* with a mix of water-soluble vitamins, including thiamine, gave improved growth in Atlantic halibut larvae (Hamre *et al.* submitted). *Artemia* contains all the other water-soluble vitamins at levels that can be assumed to be sufficient, so the increased growth was probability caused by the thiamine supplementation (Hamre *et al.* unpublished). Rotifers grown on the raw ingredients used in the present study are therefore probably deficient in thiamine and the rotifer diets should be fortified with this vitamin.

It is well known that rotifers have a requirement for high levels of vitamin B_{12} to sustain their own growth. Maruyama *et al.* (1997) found that rotifer diets should contain 1.2–1.7 mg kg^{-1} DW of cobalamin to maximize rotifer growth. This was not achieved using the YO and YA diets, while *Chlorella* contained high enough levels of cobalamin to make the YC diet sufficient in this vitamin for rotifers. The CS diet contained more than sufficient amounts of vitamin B_{12}. So, although the cobalamin content of all rotifer groups were

probably sufficient for cod larvae, diets based on yeast and fish oil or Algamac 2000 should be supplemented with vitamin B_{12} to sustain rotifer growth.

Yeast, *Chlorella* and Algamac 2000 were poor sources of vitamin E, whereas cod liver oil and Culture Selco 3000 contained enough vitamin E to bring the levels in rotifers to above both copepod levels and the requirements given for fish (NRC 1993). It is common practise in the industry to supplement diets for marine fish larvae with vitamin E at levels that are considerably higher than copepod and given requirement levels, although there is little scientific evidence to support this practise. This is exemplified by the high concentration of vitamin E in Culture Selco 3000. Kolkovski *et al.* (2000) could not demonstrate any effects of increasing vitamin E in *Artemia* from approximately 130–175 mg kg^{-1} DW, on growth, survival or stress resistance of freshwater walleye fed high levels of EPA and DHA. On the contrary, Brown *et al.* (2006) found at slight increase in growth of striped trumpeter larvae with increasing concentrations of α-tocopherol in rotifers from 114 to 1040 mg kg^{-1} DW. Marine fish larvae in culture face a higher risk of exposure to lipid oxidation products than wild fish larvae, because of the enrichment of rotifers and *Artemia* with long-chain n-3 PUFA. This may increase their requirement of vitamin E. Vitamin E is also used in the free form, as an antioxidant to protect diets and enrichment emulsions for live feed from lipid oxidation.

Yeast, Algamac 2000 and *Chlorella* were poor sources of vitamin A, and rotifers fed on these raw ingredients only, had vitamin A levels below the detection limit of our analytical method (8 μg kg^{-1}). Rotifers fed on the CS diet had vitamin A levels slightly above the assumed optimal level for young Atlantic halibut juveniles (2.4 mg kg^{-1}, Moren *et al.* 2004b). Both the inoculums, which had been grown on the Øye standard diet, and the rotifers grown on the YO diet were supplemented with cod liver oil which is fortified with vitamin A. Yet, only the innoculum had detectable levels of vitamin A. Fish oils may vary in vitamin A concentration and vitamin A may be removed during processing of the oils. Therefore, use of fish oils in rotifer diets should be accompanied by analyses of this vitamin in the rotifers. Copepods do not contain vitamin A, but the high levels of astaxanthin can probably be converted to vitamin A by the larvae at sufficient rates to cover the vitamin A requirement (Moren *et al.* 2004a). Rotifers, on the contrary, contain low levels of carotenoids and it is difficult to enrich them to obtain similar levels of carotenoids as copepods. Therefore, it is important to fortify rotifer diets with vitamin A when the dietary ingredients are poor in this vitamin.

Minerals

Of the macrominerals, phosphorus in rotifers fed yeast, *Chlorella* and Pronova oil in experiment 2, was lower than in copepods, but higher than the requirement in fish (NRC 1993). Restricted phosphorus supply within the recommended dietary range, has been correlated with vertebral deformities and mineral imbalances in Atlantic salmon (Helland *et al.* 2005) and could be an increased risk factor for bone deformities that often develop during the larval stage in farmed marine fish larvae (Cahu *et al.* 2003).

All the trace elements, except iron, were lower in all rotifer groups than in copepods. Manganese and selenium were also lower than the requirements given for fish (NRC 1993). Iron appears to be sufficient when copepod levels are used as the reference in all rotifer groups except those fed the CS diet. *Chlorella* seems to be a good source of iron, which is also supplied at a reasonable level by yeast.

Iodine in rotifers grown in the present study was slightly higher than the requirement given for fish (NRC 1993), but far below copepod levels. All dietary ingredients were poor sources of iodine. The rotifer iodine levels were slightly higher than the levels found in *Artemia* (Moren *et al.* 2006). Atlantic halibut larvae fed *Artemia* enriched with iodine to the lower range found in copepods, had increased levels of whole body thyroid hormones (Moren *et al.* 2006). Results on larval performance from this study were inconclusive, because of very high mortality of larvae during the first 1–2 weeks of the experiment (Moren *et al.* 2006). New experiments on this issue are under way in our laboratory.

Chlorella appears to be a better source of manganese than the other dietary ingredients used in this study. Nevertheless, the concentration in rotifers was only marginally increased by feeding *Chlorella*. Manganese is a candidate for supplementation to rotifer diets, because none of the rotifer groups had manganese levels above those found in copepods or those given as requirements in fish (NRC 1993). Copper in rotifers fed the diets consisting of raw ingredients, only, were below copepod levels and at the lower range of requirements given for fish. Although the complete CS diet was probably fortified with copper, the copper level in the rotifers was still below the levels found in copepods. Copepods also contain high levels of zinc, compared with all rotifer groups in the present study, which, however, contain levels above the requirements in fish. Yeast seems to be a fairly good source of zinc, but one should aim to enrich rotifers for use in culture of marine fish larvae with zinc. Selenium may be the trace element with the highest potential of being deficient in rotifers, as rotifer levels are far below the requirements in

fish. All the feed ingredients used in the present study were poor sources of selenium.

Manganese, copper and selenium are functional red-ox centres of the antioxidant enzymes superoxyd dismutase (SOD) and glutathione peroxidase (GPX), respectively (Esworthy *et al*. 1998; Lygren *et al*. 1999), where SOD handles superoxide and GPX handles both lipid and water-soluble hydroperoxides. A GPX situated in the intestinal mucosa, specifically handles fatty acid hydroperoxides from the diet and converts them to non-toxic hydroxyl fatty acid (Esworthy *et al*. 1998). Sufficient intake of these minerals is therefore important to protect the larvae against lipid oxidation and the resulting oxidation products, which can be abundant in cultured live feed enriched with n-3 PUFA.

The present study shows that rotifers are potentially deficient in several micronutrients with regard to covering the nutritional requirements of marine fish larvae. Vitamins A, C, E and thiamine and the trace elements manganese, selenium and copper may reach levels below the requirements given for juvenile and adult fish by NRC (1993). Using nutrient levels in copepods as the requirement criterion, only the B-vitamins, except thiamine, and the minerals calcium, magnesium and iron appear sufficient. The nutrient levels in rotifers are dependent on the dietary levels, and especially the B-vitamins should be checked if yeast is suspended from rotifer diets. It is important to establish enrichment techniques for the potentially limiting micronutrients in rotifers in order to develop a nutritionally balanced rotifer diet for marine fish larvae. Such techniques would also make it possible to run nutrient requirement studies with fish larvae.

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