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Marking live feeds with inert metal oxides for fish larvae feeding and nutrition studies

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Abstract

Yttrium oxide (Y_2O_3), ytterbium oxide (Yb_2O_3), lanthanum oxide (La_2O_3) and dysprosium oxide (Dy_2O_3) were evaluated as potential live feed markers for feeding and nutrition studies with fish larvae, by determining the uptake and depletion of markers over time in two trials, and quantifying ingestion of Y_2O_3-marked rotifers (*Branchionus plicatilis*) by Atlantic cod (*Gadus morhua*) in a third trial. In the first two trials, *Artemia* nauplii and rotifers quickly took up markers within 10 min to concentrations useful for nutrition studies ($>2\%$ dry weight). There was no significant difference ($P>0.05$) among temperatures in depletion of markers (10, 15, 20 °C) with *Artemia* or rotifers. Depletion from rotifers was not significantly different ($P>0.05$) between 5 and 20 min nor between 5 and 30 min for *Artemia* when marked at a concentration of 50 mg of marker per litre of seawater. In the second trial, rotifers and *Artemia* were marked with a higher concentration (250 mg L^{-1}) and allowed to deplete for a longer time (90 min). In the third trial, visual estimates of *Artemia* consumed by Atlantic cod larvae were similar to consumption estimates determined by analysis of Y_2O_3-marked *Artemia* using inductively coupled plasma optical emission spectroscopy ($r^2=0.77$).

Keywords: inert markers, consumption, microparticulate diets, rotifer, *Artemia*

Introduction

Live rotifers and *Artemia* nauplii are required feeds for most cultured temperate marine fish larvae. The production of live feeds is time and resource consuming and daily preparation requires extra staffing and expertize. Creating a nutritionally effective microparticulate diet (MPD) that would be readily accepted from the first feeding onwards could reduce the reliance on expensive and nutritionally variable live feeds (Rust, Hardy & Stickney 1993; Cahu & Zambonino-Infante 2001).

Evaluating new MPDs requires feeding and nutrition studies. Marine fish larvae readily consume rotifers and *Artemia* nauplii; therefore, these organisms are often used as controls during feeding and nutrition studies (Coutteau, Geurdan, Camara, Bergot & Sorgeloos 1997). Because good larval growth and survival can be achieved using live feeds, it has been recommended that results from MPD research be expressed relative to live feed (Bengston 1993).

Markers that can be used accurately and easily with both live feeds and MPDs are needed for fish larval feeding and nutrition studies (Teshima, Ishikawa & Koshio 2000). Austreng, Storebakken, Thomassen, Refstie & Thomassen (2000) evaluated 15 oxides (yttrium and rare-earth metals) as markers for salmonid diets and concluded that three of them, yttrium, ytterbium and lanthanum oxide, were suitable markers for digestibility trials. A fourth marker, dysprosium oxide, was less effective; however, it may be beneficial under different circumstances. The four elements are detected as bright spikes at characteristic wavelengths when analysed using inductively coupled plasma-optical emission spectroscopy (ICP-OES). These markers can be included in diets at a fraction ($\sim 1/100$) of what is required for chromic oxide for analysis (Hillestad, Asgard & Berge 1999). Chromic oxide is still commonly used in juvenile and adult fish digestibility studies; however, its value as a marker has been debated (Kabir, Wee & Maguire 1998).

Initial trials in our lab showed that yttrium oxide is quickly ingested by rotifers and *Artemia* nauplii and that Pacific cod (*Gadus macrocephalus*) and rockfish (*Sebastes* sp.) larvae will consume live feeds marked with yttrium oxide to satiation.

The current study evaluated the uptake and depletion of four inert oxides of, yttrium (Y_2O_3), ytterbium (Yb_2O_3), lanthanum (La_2O_3) and dysprosium (Dy_2O_3), by rotifers and *Artemia* nauplii to determine their suitability as markers for larval fish feeding and nutrition studies. Using ICP-OES, we measured the number of Y_2O_3-marked *Artemia* nauplii consumed by Atlantic cod larvae and compared the results with those obtained by visually counting the nauplii dissected from larval fish digestive systems.

Methods and materials

Trial 1

Marker uptake: rotifers and Artemia nauplii, low concentration

Fifty milligrams of either Y_2O_3, Yb_2O_3, La_2O_3 or Dy_2O_3 (Sigma, St Louis, MO, USA; purity 99.9%) was added to 1 L of seawater (salinity, 30 g L^{-1}) in triplicate Imhoff cones and aerated vigorously. Twelve cones (four treatments, three replicates) were set into a 30 °C water bath. After a colloidal suspension was achieved, 3.0 g (wet weight) of healthy rotifers were added to each cone. A sample was taken from each cone after time 0 and after 10, 20, 30, 60 and 90 min and placed on a 90 μm nylon screen. Each sample (∼ 200 wet mg) was rinsed vigorously with cold tap water and then dH_2O before being placed in pre-weighed glass scintillation vials. *Artemia* were marked as described above for rotifers, except that 5.0 g of *Artemia* were added to each cone.

Marker depletion

Artemia nauplii and rotifers were marked with Y_2O_3, Yb_2O_3 or Dy_2O_3 for 30 min, and La_2O_3 for 10 min, using the methods outlined above. Marked rotifers were rinsed thoroughly with cold seawater before distribution to the depletion tank. Each temperature treatment (10, 15 and 20 °C) had two replicates for a total of 24 tanks. Each tank contained 12 L of 30 g L^{-1} seawater. Approximately 1.2 g of rinsed, marked rotifers were added to each tank. Two rotifer samples (∼ wet 150 mg) were removed from each tank at 5, 10, 20 and 30 min by pouring rotifers from a cup through a clean 90 μm removable screen secured in a PVC sleeve. The sample was rinsed with clean seawater and then with dH_2O. The sample was rinsed off the screen with dH_2O into a pre-weighed scintillation vial. Samples were frozen and stored at − 10 °C before analysis. Samples for depletion of markers from *Artemia* were taken using the same methods described above for rotifers.

Artemia and rotifer survival

Subsamples of rotifers and *Artemia* (1/10 mL) were removed from each cone at 30, 60 and 90 min after the start of the trial and examined with a stereoscope. Counts of live and dead organisms were made to determine survival during the marking process.

Trial 2

Marker uptake: Artemia nauplii, high concentration

Artemia were marked as in Trial 1, except that 250 mg of marker (instead of 50 mg) was added to the marking cones and samples were removed at 15, 30, 60 and 90 min. No survival data were taken. Samples were taken on a 125 μm screen. A second uptake trial for rotifers was not performed.

Marker depletion: rotifers and Artemia nauplii

Rotifers were marked in a 250 mg marker per litre seawater solution with either Y_2O_3, Yb_2O_3, La_2O_3 or Dy_2O_3. Marked rotifers were added to the depletion tanks at 10 °C. Samples of marked organisms were taken after 1, 15, 30, 60 and 90 min. Each marker had three corresponding tanks and a sample was removed from each tank at each time. Samples were taken on a 125 μm screen. One minute was chosen as the initial sample time to reduce potential contamination by external marker not washed off. Samples for depletion of markers from *Artemia* were taken as above for rotifers.

Trial 3

Consumption

Artemia nauplii were marked for 30 min in a solution of 250 mg of Y_2O_3 L^{-1}. Marked *Artemia* were rinsed vigorously and counted into groups ($n = 5$) of 1, 50, 100 and 200 nauplii. Yttrium concentration was analysed using ICP-OES (outlined below), and the results were plotted to generate a standard equation that could then be used to determine the content of mar-

ker in an average nauplii. Atlantic cod larvae (45 dph) were allowed to feed on marked nauplii from the same batch for 30 min. Full larvae were then removed and dissected under a stereoscope to enumerate consumed *Artemia* nauplii visually. The dissected gut contents and the remainder of the larvae were placed into scintillation vials and analysed for yttrium using ICP-OES. Estimates of *Artemia* nauplii consumption by cod larvae obtained with the ICP-OES method were compared with consumption estimates obtained visually by linear regression.

Marker analysis

Marked rotifers, *Artemia* nauplii and fish larvae were ashed in a muffle furnace at 550 °C overnight and the resulting ash was digested with an aqueous mixture of 10% (v/v) hydrochloric and 10% (v/v) nitric acids for 1 h at 60 °C. Digested samples were analysed for Y_2O_3, Yb_2O_3, La_2O_3 and Dy_2O_3 using ICP-OES on a Perkin Elmer Optima 3000 Radial ICP-OES (Perkin-Elmer, Waltham, MA, USA).

Marker standards used in the analysis were formulated by Ultra Scientific, North Kingstown, RI, USA. Per cent marker concentrations were presented on a dry-weight basis.

Statistical analysis

One-way analysis of variance (ANOVA) for marker concentration and rotifer and *Artemia* survival (following arcsine transformation of proportional data) were performed to determine significant differences among treatments and time. Mean comparisons were made using Tukey's multiple comparison test at a significance level of ($P < 0.05$). Mean values per replicate were considered to be the unit of observation for statistical comparisons. Values are stated as mean SEM. The statistical software used was Prism GraphPad Software, San Diego, CA, USA.

Results

Trial 1

Marker uptake

After 90 min, rotifers had taken up markers to > 6% dry weight: from the lowest to the highest: La_2O_3 (6.95 ± 0.41%), Yb_2O_3 (8.76 ± 0.42%), Dy_2O_3 (10.94 ± 0.39%) and Y_2O_3 (12.67 ± 0.96%). After 90 min, *Artemia* had taken up markers to > 4% dry weight from the lowest to the highest: La_2O_3 (4.70 ± 0.36%), Yb_2O_3 (5.71 ± 0.36%), Dy_2O_3 (8.82 ± 0.23%), Y_2O_3 (11.17 ± 0.90%). All markers appeared to be taken up to concentrations sufficient for nutrition studies (2% dry weight) by 10 min (Table 1).

Marker depletion

There were no significant differences ($P > 0.05$) among the temperature treatments (10, 15 and 20 °C) and so all replicates were combined for comparison of depletion over time ($n = 6$). In both the *Artemia* and rotifer treatments, time zero sample values were not included for statistical comparisons because, upon inspection under the microscope, it was determined that they were contaminated with marker. Time 30 sample values were also excluded from the rotifer treatment because of contamination. Depletion of markers from rotifers marked in a 50 mg L^{-1} solution was not significant ($P > 0.05$) between 5 and 20 min (Table 1). Depletion of markers from *Artemia* nauplii marked in a 50 mg L^{-1} solution was not significant ($P > 0.05$) between 5 and 30 min (Table 1).

Uptake survival

At 90 min in the marker solution, rotifer survival was (lowest to highest) (93 ± 4%) Y_2O_3, (97 ± 4%) La_2O_3, (100%) Yb_2O_3 and (100%) Dy_2O_3. At 90 min in the marker solution, *Artemia* survival was (lowest to highest) (88 ± 3%) Dy_2O_3, (89 ± 8%) Y_2O_3, (91 ± 6%) La_2O_3, to (97 ± 3%) Yb_2O_3. There were significant differences ($P > 0.05$) for survival among markers (Table 1).

Trial 2

Marker uptake

Uptake in Trial 2 was performed for *Artemia* only. Markers were taken up quickly and to high concentrations when marked in a 250 mg L^{-1} solution La_2O_3 (14.50 ± 1.2%), Yb_2O_3 (18.71 ± 1.2%), Dy_2O_3 (24.27 ± 0.86%) and Y_2O_3 (30.33 ± 0.35%) dry weight (Table 2).

Marker depletion

Depletion of markers occurred over time for rotifers and *Artemia* when marked in a 250 mg L^{-1} solution and placed in clean seawater for 90 min. Depletion

Table 1 Uptake of markers by *Artemia* nauplii and depletion of markers from *Artemia* nauplii and rotifers

	Uptake (min)					
	0	**10**	**20**	**30**	**60**	**90**
Rotifers						
Yb	0	3.76 ± 0.36	6.78 ± 0.37	8.45 ± 0.17	11.34 ± 0.37	12.67 ± 0.96
Y	0	2.63 ± 0.30	4.27 ± 0.31	4.66 ± 0.06	6.97 ± 0.13	8.76 ± 0.42
Dy	0	4.77 ± 0.43	5.76 ± 1.34	7.77 ± 0.31	9.61 ± 0.41	10.94 ± 0.39
La	0	6.58 ± 0.97	7.8 ± 0.64	5.76 ± 0.53	6.55 ± 0.19	6.95 ± 0.41
Artemia						
Yb	0	3.40 ± 0.60	8.51 ± 0.33	10.78 ± 0.06	9.67 ± 0.80	11.17 ± 0.90
Y	0	2.97 ± 0.24	4.46 ± 0.23	4.94 ± 0.48	4.99 ± 0.33	5.71 ± 0.36
Dy	0	5.72 ± 0.57	8.06 ± 0.21	8.78 ± 0.23	7.96 ± 0.11	8.82 ± 0.23
La	0	0.56 ± 0.04	1.63 ± 0.11	2.66 ± 0.39	3.73 ± 0.40	4.70 ± 0.36

	Depletion (min)				
	0	**5**	**10**	**20**	**30**
Rotifers					
Yb	*	1.55 ± 0.06	1.37 ± 0.09	1.59 ± 0.34	*
Y		2.43 ± 0.34	2.84 ± 0.17	2.83 ± 0.27	
Dy		5.80 ± 0.45	5.53 ± 0.22	5.57 ± 0.18	
La		3.86 ± 0.56	4.50 ± 0.44	4.22 ± 0.28	
Artemia					
Yb	*	0.60 ± 0.20	0.55 ± 0.23	0.57 ± 0.34	0.60 ± 0.23
Y		0.68 ± 0.18	0.71 ± 0.20	0.66 ± 0.27	0.64 ± 0.15
Dy		0.89 ± 0.59	0.84 ± 0.30	0.80 ± 0.51	0.83 ± 0.26
La		1.37 ± 0.77	1.252 ± 0.32	1.33 ± 0.29	1.28 ± 0.98

Artemia and rotifers were marked in a solution of 50 mg marker L^{-1} of 30 °C, 30 g L^{-1} seawater for 30 min.
Values (mean ± SEM) are the per cent of marker in rotifers and *Artemia* (dry weight). Values over time in depletion treatments were not significantly different ($P > 0.05$; $n = 3$).
*Values for these treatments were excluded because they were contaminated with marker.

was less apparent in the *Artemia* treatment. Significant differences ($P < 0.05$) of concentration over time varied among markers and time (Table 2).

Trial 3

Consumption results

Marker detection was closely related to the number of *Artemia* nauplii (Fig. 1).

Consumption determined with the marker method compared favourably with visual enumeration, despite challenges in counting the number of nauplii due to their partial digestion. The relationships are described by regression equations in Figs 1 and 2.

Discussion

Simple and accurate methods for measuring feed intake by larval fish are needed for larval feeding and nutrition studies (Teshima *et al.* 2000). In our study, markers were taken up by rotifers and *Artemia* nauplii to concentrations required for use in feeding and nutrition studies with fish larvae. The detection limits of these markers are extremely low using the ICP-OES analytical technique, ranging from 2 μg L^{-1} for Y_2O_3 to 7 μg L^{-1} for La_2O_3. Assuming a rotifer dry weight of at least 300 ng per individual (Oie, Makridis, Reitan & Olsen 1997) and a marker concentration of 2%, these two markers can be detected in larvae that have been fed between four (Y_2O_3) and 12 (La_2O_3) rotifers. The marker method can quantify these markers in larvae fed formulated MPDs incorporated with 2% (dry weight) Y_2O_3 (data not shown).

The marker method can be used to make direct comparisons of live and microparticulate feeds in small fish larvae. Yttrium and Dy_2O_3 have been used in our lab successfully to compare the protein digestibility of *Artemia* and an experimental MPD consumed by 5-week-old Atlantic cod larvae (data not shown). These markers could also be used to deter-

Table 2 Trial 2. Uptake of markers by *Artemia* nauplii and uptake and depletion of markers from *Artemia* nauplii and rotifers

	Uptake (min)				
	0	**15**	**30**	**60**	**90**
Artemia					
Yb	0	27.21 ± 1.17	29.06 ± 0.98	30.16 ± 0.61	30.33 ± 0.35
Y	0	17.84 ± 0.66	18.18 ± 0.66	18.82 ± 0.73	18.71 ± 1.21
Dy	0	21.93 ± 3.61	21.47 ± 0.53	22.75 ± 0.58	24.27 ± 0.86
La	0	11.32 ± 2.59	13.56 ± 1.16	13.76 ± 1.32	14.50 ± 1.23
	Depletion (min)				
	1	**15**	**30**	**60**	**90**
Rotifers					
Yb	6.77 ± 1.12^{a}	4.53 ± 0.27^{a}	3.43 ± 0.29^{bc}	2.80 ± 0.37^{bc}	$2.00 \pm .60^{b}$
Y	4.93 ± 0.41^{a}	2.20 ± 0.19^{a}	2.70 ± 0.12^{bd}	1.80 ± 0.10^{bc}	1.40 ± 0.07^{bc}
Dy	9.97 ± 2.18^{a}	6.63 ± 0.39^{ab}	5.70 ± 0.21^{b}	4.23 ± 0.32^{b}	3.90 ± 0.35^{b}
La	5.10 ± 0.24^{a}	3.03 ± 0.83^{b}	2.10 ± 0.07^{b}	2.33 ± 0.11^{b}	2.00 ± 0.25^{b}
Artemia					
Yb	21.00 ± 0.49^{a}	17.59 ± 0.57^{bc}	16.69 ± 1.03^{b}	14.69 ± 0.22^{bd}	15.88 ± 0.40^{b}
Y	13.80 ± 0.88^{ac}	11.76 ± 0.41^{ac}	12.12 ± 0.32^{ac}	9.03 ± 1.37^{b}	10.29 ± 0.62^{bc}
Dy	17.28 ± 0.81^{a}	13.48 ± 1.02^{b}	13.39 ± 1.05^{b}	13.02 ± 0.37^{b}	12.44 ± 0.32^{b}
La	9.65 ± 0.24^{a}	8.52 ± 0.46^{abc}	8.23 ± 0.25^{bd}	7.09 ± 0.36^{bde}	6.93 ± 0.10^{bde}

Artemia and rotifers were marked in a solution of 250 mg marker per litre of 30 °C, 30 g L^{-1} seawater for 30 min.
Values (mean ± SEM) are the per cent of marker in rotifers and *Artemia* (dry weight). Values in the same row with different superscripts are significantly different ($P < 0.05$).

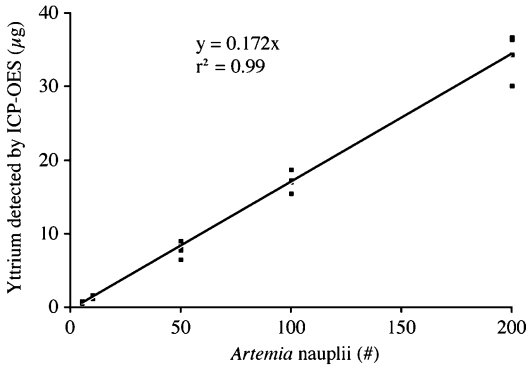

Figure 1 Linear regression of marker concentration in *Artemia* nauplii for enumeration equation. *Artemia* were marked in a solution of 250 mg L^{-1}, 30 °C, 30 g L^{-1} seawater and counted into groups of 1, 10, 50, 100 and 200 ($n = 5$ for each group).

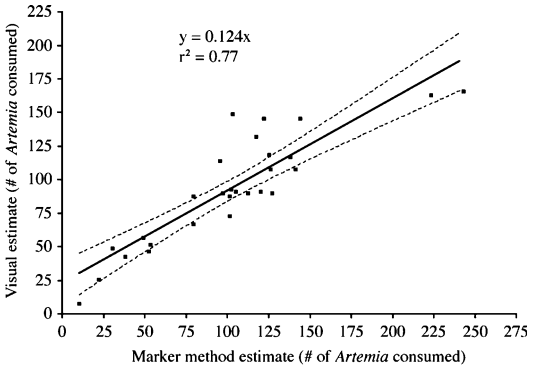

Figure 2 Linear regression of estimates of *Artemia* nauplii consumption by cod larvae obtained with the ICP-OES method compared with consumption estimates obtained visually.

mine the acceptability of unique diets marked with different markers or different sizes of the same diet. Otterå, Garatun-Tjeldsto, Julshamn and Austreng (2003) state that when Y_2O_3, Yb_2O_3, La_2O_3 and Dy_2O_3 were added to the same diet and fed simultaneously to cod juveniles, similar ingestion rates resulted.

In Trial 1, for both the rotifers and *Artemia* nauplii, marker concentration over time among the three temperature treatments (10, 15 and 20 °C) was not significantly different. Time in clean seawater did not influence marker concentration between 5 and 20 min for rotifers when marked at 50 mg L^{-1}. Depletion was not significantly different between 5 and

30 min for *Artemia*; however, some markers were depleted over a longer time in Trial 2. However, this depletion should not significantly influence trial values if the feeding period is short (5–30 min) or the depletion rate is accounted for during the feeding portion of a trial. Ideally, during a feeding trial, fish larvae would consume marked feeds quickly after introduction into the tanks. However, it may take time for larvae to begin feeding and then more time for fish to become satiated. One way to account for depletion would be to add marked feeds to replicates with no fish at the start of a trial. Samples of feeds could be removed several times throughout the trial to obtain a depletion curve of concentration of marked feeds. An average could be used for most calculations, assuming that fish were consuming feed at the same rate during the trial. This could be easily determined before a feeding trial for each species and rearing procedure.

Observation of rotifers and *Artemia* nauplii under a stereoscope after marking showed that they evacuated large faecal pellets containing the markers that clumped together with other pellets forming larger masses. Large marked faecal clumps may not have passed through the 90 μm screen. Thus, the marker may have been retained in some initial samples in Trial 1, yielding an artificially high time 0 value. This contamination resulted in a large decline in apparent concentration between 0 and 5 min (> 50% loss in 5 min for rotifers). For Trial 2, we collected the initial sample after 1 min in the depletion tanks, anticipating that if any marker remained on the organisms it would be washed off. We also increased the mesh size of the screen from $\sim$ 90 to $\sim$ 125 μm. Although steps were taken in Trial 2 to reduce contaminated samples, there was still a significant decline in concentration from 1 min (t_1) to 15 min (t_{15}) in *Artemia* nauplii for Yb_2O_3 and Dy_2O_3. There was a decline in concentration for La_2O_3 and Y_2O_3 during the trial; however, it was not significant ($P > 0.05$). The decline in concentration among markers from t_1 to t_{15} was more apparent for rotifers. This drop in concentration from t_1 to t_{15} may still be an artefact of the marking process and may not represent the actual amount ingested by rotifers and *Artemia* nauplii. It may be beneficial to rinse marked feeds in clean mildly aerated seawater for 15 min after marking to ensure that the external marker contamination is rinsed off and marker waste settles. Several steps can be taken to ensure that rotifers and *Artemia* are sufficiently marked before a trial. First, for *Artemia*, inspect nauplii before marking to make sure that the gut is functional. Keep organisms in the marker solution for at least 30 min and aerate aggressively to keep the markers in solution. View marked organisms under a dissecting microscope to confirm that marker has been consumed before continuing with the trial. Finally, rinse the marked live feeds thoroughly using the largest screen possible to wash out marker and faecal material containing markers, and recheck with the microscope.

In general, *Artemia* and rotifers marked with Yb_2O_3 and Dy_2O_3 had the highest concentration and *Artemia* and rotifers marked with La_2O_3 and Y_2O_3 had lower concentrations. We did not compare among markers statistically because uptake and depletion differences among the markers may have been related to marker density and/or particle size differences rather than gut physiology. Follow-up studies are needed to determine the impact of particle size and density on marker uptake.

There were no significant differences ($P > 0.05$) in survival among marker treatments. Survival for *Artemia* (88–97%) after 90 min of marking was generally lower than survival for rotifers (93–100%). However, each *Artemia* marker treatment, except Dy_2O_3, had at least one replicate with 100% survival after 90 min.

When marked in a solution of 50 mg L^{-1}, depletion may be less than when organisms are marked with 250 mg L^{-1}. One to 3% of marker per dry weight of live feed is a good target concentration. In our lab, we include markers at a level of 2% dry weight in our larval diets for feeding and nutrition experiments.

Enumerating live feeds consumed using the marker method may be more accurate than visual counting because digestion can make live feeds difficult to distinguish. The marker method is a more practical means of quantifying feed consumption when a large sample number is required. Quantifying consumption visually is tedious and time consuming. Future work will include using the marker method to determine the apparent digestibility of live and artificial diets, and the acceptability of marked live feeds.

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