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Evaluation of wax spray beads for delivery of low-molecular weight, water-soluble nutrients and antibiotics to *Artemia*

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ABSTRACT

Culture of the early larval stages of many marine fish species is often characterized by high larval mortality and poor growth. Comparison of the nutritional composition of cultured live prey, such as species of rotifers and *Artemia*, with that of "natural" prey species, such as copepods, indicates possible deficiencies in essential micronutrients that could explain the poor performance of larvae fed on these cultured foods. Enrichment of live prey species by feeding them on artificial diets is difficult due to high leakage losses from microparticles in aqueous suspension. In this study, wax spray beads (WSB) consisting of bees wax, with or without a 5% *w*/*w* extract of a synthetic marine phospholipid (mPHL), were evaluated for their potential for delivery of water-soluble micronutrients and the antibiotic oxytetracycline (OTC) to *Artemia*. The micronutrient mix consisted of amino acids, vitamins and trace minerals and was based on the nutritional composition of copepods. Leaching experiments indicated that nitrogen (N) retention of WSB containing the micronutrient mix rapidly declined in the first 5 to 10 min of suspension in seawater; however, subsequent N losses over a 2 h period occurred at much lower rates, resulting in overall N retention efficiencies of 44% and 24% for WSB prepared with either 100% wax or 95% wax/5% mPHL, respectively. Substantial losses of labile micronutrients, such as vitamin A, vitamin E, astaxanthin, selenium, vitamin C and thiamin, occurred during WSB preparation. Furthermore, production of peroxidation products and losses of astaxanthin were greater with beads containing 5% mPHL compared with beads prepared with 100% bees wax. *Artemia* ingested and broke down riboflavin-containing WSB prepared with 100% bees wax and liberated riboflavin was observed in their digestive systems. *Artemia* fed OTC-containing WSB prepared with 100% bees wax retained OTC after purging their guts of ingested WSB. WSB may be useful in delivering water-soluble substances to *Artemia* as well as other marine suspension feeders capable of breaking down these beads by mechanical or enzymatic processes. The advantage of using wax as the lipid carrier, compared with phospholipid and triacylglycerol lipid types, is that it is relatively inert and will less likely react with incorporated materials to produce peroxides or result in excessive contributions to the lipid nutrition of the target organism.

1. Introduction

Presently, the early larval stages of many marine fish and crustacean aquaculture species are raised on live feeds, such as *Artemia* and rotifers (Støttrup and McEvoy, 2003). These live feeds are often of variable quality (Person Le Ruyet et al., 1993) and differ in nutritional composition from natural prey items, such as copepods (Table 1), perhaps explaining reported inferior performance of species fed these feeds compared with natural prey (Shields et al., 1999; Evjemo et al., 2003; Drillet et al., 2006; Hamre, 2006; Rajkumar and Kumaraguru vasagam, 2006; Seoka et al., 2007).

Live prey, such as *Artemia* and rotifers, have been nutritionally enriched by being fed algae, yeast and lipid-emulsion based formulations (Sorgeloos et al., 2001; Muller-Feuga et al., 2003; Lie et al., 2006; Srivastava et al., 2006). Delivery of low-molecular weight, water-soluble nutrients, such as amino acids and water-soluble vitamins, using artificial particles has proven difficult because of rapid leaching losses (Langdon, 2003; Kvåle et al., 2006). Liposomes have been reported to successfully deliver amino acids to *Artemia* (Hontoria et al., 1994; McEvoy et al., 1996; Tonheim et al., 2000; Monroig et al., 2003, 2006, 2007a) and Touraki et al. (1995, 2001) have used liposomes for delivery of water-soluble antibiotics to *Artemia*. However, production of liposomes usually depends on the use of expensive purified phospholipids and involves multiple steps in the preparation process, making these particle types both expensive and difficult to make on a large scale.

Researchers have investigated the use of lipid spray beads to deliver water-soluble substances to marine suspension feeders (Buchal and Langdon, 1998; Önal and Langdon, 2004a,b; Nordgreen

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Table 1
Reported range of concentrations of potentially limiting micronutrients in enriched and non-enriched rotifers and *Artemia* nauplii, compared with those of copepods and other natural zooplankton prey species for marine fish larvae

Nutrients	Rotifers	*Artemia* nauplii	Copepods and zooplankton
	Nutrient concentrations (μg g^{-1}; dry wt.)		
Iodine	3–8	0.5–4.6	50–350
Manganese	4–5	4–30	8–25
Copper	3–8	7–40	12–38
Zinc	62–64	120–310	340–570
Selenium	0.08–0.09	2.2	2–5
Iron	57–114	63–130	85–371
Riboflavin	23–43	30–60	14–35
Thiamin	2–125	6–21	13–46
Vit C	181–576	361–690	38–1106
Vit E	85–889	100–500	23–209
Carotenoids	4–16	650–750	321–1422
Vitamin A	0.5–5	0	0.03–0.18
Free amino acids:			
Taurine	50–560	7225–8188	5563–19,625
Arginine	2368–4526	2143–2387	9354–19,737
Methionine	89–1028	15–134	104–298

Micronutrient compositions for rotifers, *Artemia* and copepods were obtained from Mæland et al. (2000), Van der Meeren (2008) and Hamre et al. (2006, 2008).

et al., 2007). These beads can be easily produced using a simple spray technique whereby a mixture of molten lipid and core material is sprayed into a cooled chamber to harden the lipid matrix and trap the core material in the beads. Previous research on lipid spray beads has focused on evaluating the performance of beads prepared with methyl palmitate (Önal and Langdon, 2004a), triacylglycerols (Buchal and Langdon, 1998; Önal and Langdon, 2004b) and phospholipids (Nordgreen et al., 2007). These studies indicated that spray beads prepared with these lipid types retained amino acids and other water-soluble materials better than most other types of microparticles, although substantial leakage losses still occurred (Langdon, 2003).

In this study, we examined the use of spray beads prepared with bees wax for delivery of water-soluble substances to *Artemia*. We chose bees wax as a potentially useful lipid matrix because 1) it is inexpensive, 2) it has a high melting point (mp 61–65 °C; Sigma–Aldrich, Leirdal, Norway) it remains hard at typical aquaculture temperatures, 3) it is more hydrophobic than other lipid classes, with the possibility of good retention of water-soluble substances, 4) it is relatively inert compared with other lipid classes and less likely to interact with core materials or make excessive contributions to the lipid nutrition of target organisms. We also tested the effects of small additions of extracted synthetic marine phospholipid (mPHL; Omegalec A2; Natural, Lysaker, Norway) to improve the dispersibility of WSB and as a source of possibly essential *n*-3 polyunsaturated fatty acids (PUFA) and phospholipids (Geurden et al., 1997; Sargent et al., 1999; Cahu et al., 2003).

We first examined the effects of both the preparation process and aqueous suspension of beads on the composition of an incorporated micronutrient mix and formation of lipid peroxidation products. Secondly, we carried out feeding experiments to determine the ability of *Artemia* to ingest and break down wax spray beads (WSB) and retain core material (oxytetracycline) delivered by the beads.

2. Materials and methods

2.1. Preparation of WSB

2.1.1. Preparation of marine phospholipid (mPHL) extract

One hundred grams of a commercial product (Omegalec A2; Lysaker, Norway), composed of a synthetic marine phospholipid dissolved in 36% marine triacylglycerol, was extracted three times with 500 ml cold (2 °C) acetone under N to reduce its triacylglycerol content. The acetone phase, containing triacylglycerol, was separated from the lower phospholipid-enriched phase after 8 h settlement at 2 °C. Residual acetone was removed from the phospholipid extract by bubbling with nitrogen (N) at 50 °C, followed by freeze-drying for 48 h.

The semi-purified synthetic mPHL extract was a hard, dark brown material at room temperature, consisting of 85% phospholipid and 15% neutral lipid (Table 2). Preliminary experiments indicated that the mPHL extract could not be melted by heat to allow formation of WSB by spraying; however, one part by weight of mPHL could be dissolved with heating (95 °C) in one part bees wax to create beads composed of up to 50% *w*/*w* mPHL extract.

2.1.2. Preparation of a micronutrient mix

A micronutrient mixture, composed of fat-soluble nutrients as well as water-soluble vitamins, minerals and amino acids, was prepared and incorporated in WSB (Table 3). The composition of the mixture was based on that of naturally occurring zooplankton prey species (mainly copepods) for marine fish larvae (Mæland et al., 2000; Van der Meeren, 2008; Hamre et al., 2006, 2008) and focused on nutrients that might be limiting in cultured prey species, such as rotifers and *Artemia* (Table 1). Free amino acids were included because researchers have suggested that they may be important in the nutrition of early fish larvae with limited proteolytic digestive capabilities (Dabrowski and Rusiecki, 1983; Rønnestad et al., 1999; Applebaum and Rønnestad, 2004). We chose to include methionine, arginine and taurine as representative amino acids because concentrations in live prey are potentially growth-limiting for marine fish larvae (Helland et al., 2003; Van der Meeren, 2008; Kim et al., 2005).

In order to ensure consistent concentrations of water-soluble micronutrients among beads, the nutrients were first co-dissolved in distilled

Table 2
Lipid class and major fatty acid percent compositions of lipids used to prepare wax spray beads (WSB) as well as of WSB before and after suspension in seawater for four minutes

	Lipid mixtures			WSB			
				Pre-leached		Post-leached	
	Bees wax	mPHL	95% wax+ 5% mPHL	100% wax	95%wax/+ 5% mPHL	100% wax	95%wax/+ 5% mPHL
Neutral lipids	95.35	15.02	84.1	97.68	89.82	97.51	87.69
Phospholipids	4.66	84.99	15.9	2.32	10.18	2.49	12.32
Fatty acids							
16:0	37.05	10.86	33.71	37.43	38.13	37.47	36.66
18:0	11.69	2.44	12.71	8.35	8.17	11.53	11.19
18:1*n*-9	4.41	6.31	6.49	5.10	5.25	4.81	4.94
18:1*n*-7	0.00	0.97	0.17	0.00	0.15	0.00	0.12
18:2*n*-6	0.15	35.38	2.88	0.25	1.77	0.24	1.57
20:0	6.00	0.78	4.85	5.91	5.70	5.81	5.57
18:3*n*-3	0.12	4.43	0.49	0.00	0.00	0.00	0.26
20:1*n*-9	0.29	1.53	2.53	1.63	1.71	1.59	1.66
22:0	7.78	0.92	5.41	7.51	7.24	7.43	7.12
22:1*n*-9	0.00	1.31	0.98	4.01	3.38	0.51	0.57
20:4*n*-3	3.91	0.53	2.98	0.00	0.07	0.05	3.70
20:5*n*-3	0.86	6.98	0.44	0.00	0.25	0.00	0.17
24:0	9.09	0.56	6.31	8.92	8.36	8.79	8.39
22:5*n*-3	0.23	5.12	0.67	0.16	0.37	0.22	0.35
22:6*n*-3	0.00	10.98	0.94	0.00	0.53	0.00	0.34
Sum saturated	71.61	15.56	63.00	68.11	67.60	71.03	68.93
Sum monoenes	4.71	10.12	10.17	10.74	10.49	6.91	7.31
Sum polyenes	5.27	63.43	8.39	0.41	2.99	0.50	6.39
Sum *n*-3	5.12	28.04	5.51	0.16	1.21	0.27	4.82
Sum *n*-6	0.15	35.38	2.88	0.25	1.77	0.24	1.57
n-3/*n*-6	34.92	0.79	1.98	0.63	0.69	1.12	3.09

Two samples each were analyzed for bees wax, marine phospholipid extract (mPHL) and pre-leached beads; four samples analyzed for each post-leached bead type.

Table 3
Expected and measured concentrations of micronutrients in both a nutrient mix as well as pre- and post-leached wax spray beads (WSB)

	Salts/compounds used to prepare nutrient mix	Expected conc. in nutrient or lipid fraction	Measured conc. in nutrient or lipid fraction	Expected conc. in WSB	Measured conc. in pre-leached WSB		Measured conc. in post-leached WSB	
					100% wax	95% wax/5% mPHL	100% wax	95% wax/5% mPHL
		Nutrient concentrations (μg g^{-1}; dry wt.)						
Iodine	KI	39,954	20,525	2642	2319	2153	1217	513
Manganese	$MnSO_4{\cdot}4H_2O$	2854	1938	189	202	210	128	66
Copper	$CuSO_4{\cdot}5H_2O$	4566	2501	302	303	315	238	155
Zinc	$ZnSO_4{\cdot}7H_2O$	65,069	30,643	4302	3230	3392	3318	3423
Selenium	Na_2SeO_4	571	0	38	0	0	0.0	0.0
Iron	$FeSO_4{\cdot}7H_2O$	42,237	21,863	2793	2522	2629	2163	1550
Riboflavin		11,416	7107	755	1549	981	1292.5±20.0[a]	581.7±23.4[b]
Thiamin	Thiamin monophosphate·$2H_2O$	5708	1045	377	66	99	42.4±2.8[a]	26.4±2.1[b]
Vit C	Stay-C 35, Rovimix[1]	114,155	7018	7548	0	0	0.0±0.0	0.0±0.0
Vit E	α-Tocopheryl acetate	9699	3420	9058	3242	3222	3039±33.2[a]	3244±35.2[b]
Astaxanthin		909	ND	849	969	132	671±140[a]	8.5±1.4[b]
Vitamin A	Retinyl acetate	29	9	27	6	4	6.3±0.2[a]	3.9±0.6[b]
Taurine		285,388	218,018	18,869	25,200	24,400	13,933±315[a]	5693±57.0[b]
Arginine		285,388	190,494	18,869	21,100	20,200	10,786±378[a]	4406±66.0[b]
Methionine		142,694	111,906	9435	11,800	10,900	5693±110[a]	2060±93.0[b]

Concentrations of nutrients remaining in leached WSB (after 4 min aqueous suspension) were statistically compared between bead types (Tukey/Kramer test) and significantly ($P<0.05$) different concentrations denoted with different letters.
Trace metals and their salts were obtained from Merck, Darmstadt, Germany. All other nutrients, except vitamin C, were obtained from Sigma–Aldrich, St. Louis, USA.
[1]DSM Nutritional Products, Basel, Switzerland. ND — not determined.

water, followed by freeze-drying and grinding in an atmosphere of dry N. The water-soluble nutrient mix was prepared by first dissolving the amino acids in distilled water with stirring at 60 °C, followed by addition of the trace metal mix. The solution was cooled to 20 °C, water-soluble vitamins and KI were added to make up a final nutrient concentration of 2.2% *w*/*v* and the solution was then freeze-dried for 72 h. After freeze-drying, powdered vitamin C (Stay-C 35; DSM Nutritional Products, Basel, Switzerland) was added. The mixture was ground for 1–2 h with a pestle and mortar under dry N (Fritsch Mortar Grinder Pulverisette 2, Idar-Oberstein, Germany) and finally stored at − 80 °C under dry N in the dark.

Microscopic examination of the final ground micronutrient mix indicated that some large (>10 μm) crystals remained that could not be eliminated with further grinding. To ensure homogeneity of the beads' nutrient composition, distilled water was added to the mix to dissolve the crystals, resulting in a 33% *w*/*v* aqueous slurry of <2-μm micronutrient particles that was used for bead preparation.

2.1.3. Formation of WSB

WSB were prepared following methods described by Önal and Langdon (2004a,b). Bees wax (refined; Sigma–Aldrich, Leirdal, Norway) was melted at 95 °C and marine PHL extract was dissolved in the molten wax for preparation of beads containing mPHL. Lipid-soluble vitamins and astaxanthin were then dissolved with sonication (Vibra Cell, Sonica Materials, Danbury, CT, USA) in the molten lipid. A 33% *w*/*w* aqueous slurry of water-soluble micronutrients (Table 3) was then added to the molten lipid mixture at a ratio of 1:4 lipid by weight. The mixture was sonicated until a stable emulsion was formed. The emulsion was then poured into a heated chamber and sprayed into a stainless-steel cylinder that was chilled with liquid N vapor. N was used as the pressurized gas for spraying. The cooled and hardened lipid beads were collected and stored at − 80 °C under N until used.

In preliminary experiments, WSB made up of a mixture of bees wax and from 0 to 50% *w*/*w* mPHL were prepared to determine the effect of different concentrations of mPHL on N retention and bead dispersibility. N retention decreased with increases in mPHL concentration while addition of as little as 5% mPHL improved aqueous dispersion. Based on these results, WSB prepared with bees wax plus 5% mPHL were subsequently compared with WSB prepared with 100% wax for micronutrient delivery.

2.2. Characterization of WSB with incorporated micronutrient mix

2.2.1. Size

The diameters of 30 beads of each type were randomly measured microscopically (Olympus, BX51, Oslo, Norway) using a calibrated image analysis system (Olympus DP-SOFT Version 3.2, Oslo, Norway).

2.2.2. Nitrogen retention efficiencies of WSB suspended in seawater

The mean N content of triplicate samples of bees wax, mPHL and micronutrient mix was initially determined using a N analyzer (Leco FP-528, St. Joseph, MO, USA). N analyses alone could not determine the relative contributions of micronutrients and lipids to N loss from beads suspended in seawater because the mPHL extract contained N. Therefore, total N retention efficiencies (RE) for WSB were determined (in quadruplicate) by calculating percent change in total N content after suspension of WSB in seawater for a known period of time and expressed as:

$$\mathrm{RE\ (total\ } N) = \frac{\text{Total } N \text{ content after SW suspension}}{\text{Total initial } N \text{ content}} \times 100$$

Weighed samples of about 100 mg WSB were added to screw-capped 20 ml test tubes filled with 10 ml of 0.5% *w*/*v* sodium dodecyl sulfate (SDS) made up in chilled (5 °C), 0.45 μm-filtered seawater (35‰). Addition of SDS was reported by Nordgreen et al. (2007) to improve bead dispersion and reduce the effects of clumping on retention efficiency measurements without affecting lipid wall permeabilities. The suspension was then mixed vigorously for 2 min using a Vortex Genie (Scientific Industries, New York, NY, USA) and placed on a shaker table (SM, Edmund Bühler, Tübingen, Germany) at 20–22 °C. After pre-determined suspension periods, beads were filtered onto N-free, 0.64 μm Durapore membrane filters (25 mm; Millipore, Billerica, MA, USA), rinsed with 10 ml of chilled (5 °C) filtered seawater then frozen at − 20 °C until N analysis.

2.2.3. Micronutrient retention by WSB suspended in seawater

Micronutrient content and retention by beads were determined within 3 weeks of bead preparation. Retention of each of the micronutrients was determined for beads after 4 min suspension in 0.5% *w*/*v* SDS made up in seawater, as described for determination of N retention efficiencies. The micronutrient content of two weighed (250–500 mg) freeze-dried samples of each bead type was analyzed before and four samples were analyzed after bead suspension in seawater. All WSB samples from retention experiments were freeze-dried and stored at − 80 °C under nitrogen until they were analyzed. In addition, the micronutrient composition of the mix before incorporation in WSB was determined as well as lipid class, fatty acid and fat-soluble vitamin composition of WSB.

2.2.4. Analysis of micronutrients

Trace metals and iodine concentrations were measured directly in WSB samples without pre-extraction with organic solvents. Freeze-dried samples of WSB were wet digested using a microwave technique with nitric acid and 30% hydrogen peroxide (Julshamn et al., 2000). The samples were then analyzed for Fe, Mn, Cu, Zn and Se (Julshamn et al., 2004) and iodine (Julshamn et al., 2001).

Known dry weights of WSB (200–500 mg) were extracted with chloroform/methanol according to the method of Bligh and Dyer (1959) in order to determine concentrations of other micronutrients and lipids. The chloroform phase of extracted WSB was collected, then the chloroform removed and the lipid extract re-constituted in a known volume of solvent for analysis of astaxanthin, vitamins E and A, lipid classes and fatty acids. Vitamins A and E were determined by normal phase HPLC after saponification and extraction with hexane (Lie et al., 1994; Moren et al., 2004). Extraction and analyses of astaxanthin were conducted as described by Torstensen et al. (2004). Lipid class composition was determined by high performance thin layer chromatography (HPTLC) as described in Jordal et al. (2007) and Bell et al. (1993). Lipids were extracted with chloroform/methanol, applied to the HPTLC plate and developed in two phases to separate phospholipid classes and neutral lipids. The lipid classes were visualized by charring and identified by comparison with external standards and quantified by scanning densitometry. Fatty acids were analyzed according to methods described by Lie and Lambertsen (1991).

The aqueous-methanol phase of the Bligh and Dyer (1959) extraction was collected, freeze-dried and then re-constituted in distilled water for analysis of water-soluble vitamins and amino acids. WSB aqueous extracts were treated with acid phosphatase to remove phosphate from the ascorbate-2-phosphate (Stay-C 35; Hamre et al., 2006). Ascorbic acid was then analyzed by HPLC with an amperiometric electrochemical detector as described by Mæland and Waagbø (1998). Riboflavin was analyzed using semi-automated microbiological assays as described by Mæland et al. (2000). Thiamin analysis was carried out by HPLC according to CEN (2002) and free amino acids were determined according to methods described by Srivastava et al. (2006).

2.2.5. Lipid peroxidation products

WSB were analyzed for lipid peroxidation products by measuring thiobarbituric acid-reactive substances (TBARS) before and after suspension in seawater for 4 h at room temperature (20–22 °C), according to the method described by Hamre et al. (2001).

2.3. Ingestion and retention of core material by Artemia fed on 100% WSB

2.3.1. Artemia culture

Artemia cysts (GSL, Salt Creek Inc., Aquatic Ecosystems, FL, USA) at 1 g l^{-1} were added to 1-μm filtered, autoclaved seawater (33‰, 26 °C) in 2 l flasks and incubated for 24 h. After hatching, nauplii were transferred to flasks containing 1-μm filtered, autoclaved seawater. Two-day old, unfed metanauplii were used in feeding experiments.

2.3.2. Breakdown of ingested 100% WSB

WSB were prepared with 100% bees wax and ground riboflavin (Önal and Langdon, 2004a) in order to visualize their ingestion and breakdown by *Artemia*. WSB were fed to *Artemia*, with gentle agitation to maintain the beads in suspension. After approximately 1 h of feeding, *Artemia* were sampled and examined under a Leica DM1000 (Leica Microsystems, Wetzlar, Germany) fitted with a Spot Insight QE color camera (Diagnostic instruments, Sterling heights, MI USA) epifluorescent microscope under a 40×/0.65 objective (excitation 450–490 nm, emission 515–528 nm) for evidence of breakdown of ingested beads and release of riboflavin into their digestive systems. Bead diameters were measured from digital images using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA).

2.3.3. Retention of core material (oxytetracycline) by Artemia fed on 100% wax WSB

In order to demonstrate uptake and retention of released core material from WSB prepared with 100% bees wax, 2-day old *Artemia* metanauplii were fed beads containing the antibiotic oxytetracycline (OTC). OTC was chosen as a marker for this purpose instead of a nutrient in order to avoid *Artemia* potentially rapidly metabolizing and reducing retained marker concentrations (Dhont and Van Strappen, 2003). OTC is also an approved (US Food and Drug Administration, 2007) therapeutic drug for treating diseases in cultured aquatic organisms. The 100% wax beads containing 7.7% *w*/*w* OTC were prepared as described above and stored at 5 °C.

Metanauplii (approx. 230 ind. ml^{-1}) were cultured in 50 ml centrifuge tubes containing 25 ml of 1-μm filtered, autoclaved seawater (26 °C, 33‰). The tubes were placed on a Glas-col® Rugged Rotator (Terre Haute, IN, USA) at 30 rpm. Treatments consisted of additions of one of the following to a set of six replicated *Artemia* cultures each: 1) 0.025 g 100% wax beads containing OTC, suspended in 1 ml of 1% *w*/*v* gum arabic solution, 2) a solution of 0.002 g ml^{-1} OTC dissolved in 1 ml of 1% *w*/*v* gum arabic solution to equal concentrations of OTC added within beads, 3) 1 ml of 1% *w*/*v* gum arabic solution only. Gum arabic was added to facilitate dispersion of the beads. *Artemia* densities were not different among treatments (two-way ANOVA; $P = 0.78$). *Artemia* were fed the experimental substrates at time 0 and every 6 h over a period of 18 h.

After 22 h, *Artemia* were collected on a 105 μm sieve and transferred to clean test tubes containing 25 ml 1-μm filtered, autoclaved seawater (26 °C, 33‰). At 23 h, *Artemia* in three out of the six replicate test tubes per treatment were fed 50 μl algae (Shellfish Diet 1800™ Aquatic Ecosystems, Apopka, FL, USA) while the other three cultures were maintained in filtered seawater for the remaining 2 h. *Artemia* cleared their guts of WSB after feeding on algae for 2 h while the guts of those maintained in filtered seawater still retained small amounts of beads. *Artemia* from each replicate were then collected on a 105 μm sieve and transferred to 3 ml seawater and stored at 5 °C in the dark until OTC analysis. *Artemia* densities of each replicate sample were determined using a Sedgewick–Rafter Counter.

OTC concentrations in *Artemia* were determined by measuring the effect of whole body extracts on growth of *Escherichia coli* cultured in Luria–Bertani (LB) both (5 g l^{-1} NaCl, 10 g l^{-1} Bacto™ tryptone and 5 g l^{-1} Bacto™ yeast extract). A volume of 500 ml LB broth was inoculated with *E. coli* 3 h prior to addition of OTC extracts. At 2.5 h, 4 ml aliquots of inoculated LB broth were transferred to 20 ml sterile test tubes. *Artemia* in each replicate were broken down in 3 ml seawater using a B. Braun Labsonic® L sonicator at high power for 30 s. Then 1.5 ml aliquots of tissue homogenate from each replicate were transferred to microcentrifuge tubes and centrifuged (Beckman Microfuge E) at 12,500 ×g for 15 min. One milliliter of supernatant from each replicate was removed, filtered through a 0.2 μm cellulose acetate Nalgene® syringe-filter into a 20 ml test tube containing the inoculated LB broth and incubated for 24 h at 43 °C. Triplicate OTC standards were prepared in seawater at 0, 3.9, 15.6, 62.5 and

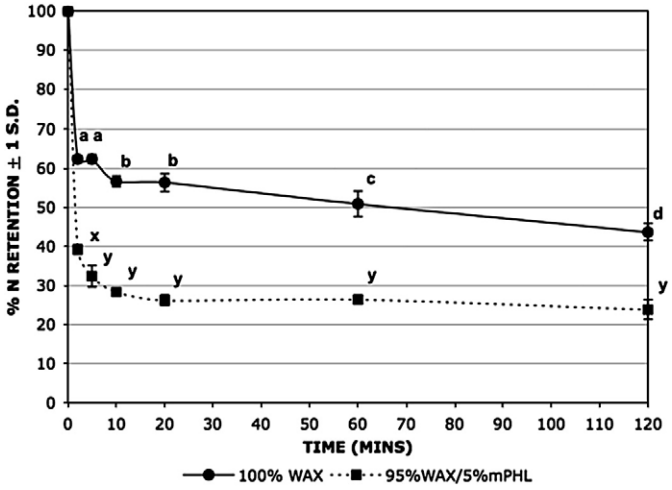

Fig. 1. Nitrogen retention of wax spray beads (WSB) containing a micronutrient mix (Table 2) and prepared with either 100% bees wax or 95% bees wax and 5% marine phospholipid extract (mPHL). WSB were agitated at 20–22 °C in 0.5% *w*/*v* sodium dodecyl sulfate solution made up in seawater and WSB samples ($n=4$) were taken and analyzed over a 2 h period. Nitrogen retention values for each bead type that are significantly different (Tukey/Kramer test; $P<0.05$) are labeled with different superscript letters.

250 μg ml^{-1}. One milliliter aliquots of standard solutions were added to test tubes containing inoculated LB broth and incubated for 24 h at 43 °C.

Absorbance due to bacteria density was measured spectrophotometrically at 600 nm. A standard curve was calculated with known additions of OTC (see above) that yielded the formula:

$$y = -0.0035x + 0.2271$$

where y was the absorbance of a given sample due to bacterial density and x was the concentration of OTC (μg ml^{-1}) ($r^2 = 0.9744$; $P = 0.0115$).

2.4. Statistics

The suitability of data for analysis by ANOVA was examined by viewing normal probability plots of residuals to determine homogeneity of variances. If necessary, percentage values were arc-sine transformed to meet assumptions of ANOVA. Tukey/Kramer multiple range tests were carried out when ANOVA indicated a significant ($P<0.05$) overall treatment effects. Statistical analyses were carried out using Statview (SAS, version 5.01) and Statgraphics (Starpoint, Inc., version 5.0).

3. Results

3.1. Composition of pre-leached wax spray beads

3.1.1. Lipids

The bees wax was composed of 95% neutral lipid and 5% phospholipid while the mPHL extract contained 15% neutral lipid and 85% phospholipid (Table 2). Bees wax and mPHL also differed in fatty acid composition, with bees wax consisting of 72% saturated fatty acids while the mPHL extract contained only 16% saturated fatty acids. Polyenes were the most abundant fatty acid type (63% *w*/*w*) in the mPHL extract, with 20:5*n*-3 and 22:6*n*-3 present at 7 and 11% *w*/*w*, respectively. Bees wax had a relatively high concentration of 4% 20:4*n*-3 compared with other polyenes that resulted in a high *n*-3/*n*-6 ratio of 35.

Concentrations of unsaturated fatty acids declined from 19 to 13% *w*/*w* and saturated fatty acids increased from 63 to 68% *w*/*w* as a result of preparation of 95% wax/5% mPHL WSB, with losses of both 20:5*n*-3 and 22:6*n*-3 fatty acids. Concentrations of saturated fatty acids did not change as a result of preparing 100% wax beads but there were changes in the concentrations of unsaturated fatty acids, with 20:4*n*-3 decreasing from 4 to 0% while 22:1*n*-9 increased from 0 to 4% *w*/*w*.

3.1.2. Micronutrients

Measured concentrations of micronutrients in the mix were generally lower compared with expected concentrations (Table 3). During preparation of the mix, there were substantial losses of 100% selenium, 94% vitamin C (Stay-C 35) and 82% thiamin as well as high losses of vitamin A (69%) and vitamin E (65%) (astaxanthin was not determined).

After combining losses due to the preparation of both the micronutrient mix and beads, we found that measured and expected concentrations of amino acids and trace metals (apart from selenium) differed by ±22% in pre-leached beads (Table 2). In contrast, measured concentrations of riboflavin were up to 95% higher than expected in pre-leached 100% wax beads, even though a loss of 38% occurred as a result of preparing the micronutrient mix. Substantial losses of vitamin C occurred during preparation of both the micronutrient mix and beads, resulting in an overall 100% loss. Losses of vitamins A and E mainly occurred during preparation of the lipid mixture. There

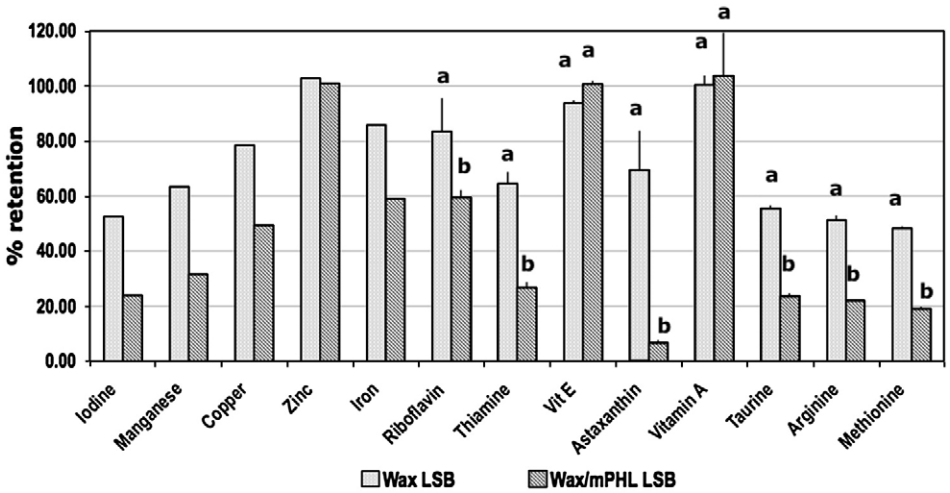

Fig. 2. Retention of micronutrients by wax spray beads (WSB) prepared with either 100% bees wax or 95% bees wax and 5% marine phospholipid extract (mPHL). WSB samples ($n=2$ for metals and iodine; $n=4$ for other micronutrients) were taken and analyzed after WSB were agitated for 4 min in 0.5% *w*/*v* sodium dodecyl sulfate solution made up in seawater at 5 °C. Values for each micronutrient that are significantly different between bead types (Tukey/Kramer test; $P<0.05$) are labeled with different superscript letters.

was an 84% loss of astaxanthin in beads prepared with 95% wax/5% mPHL but little loss in beads prepared with 100% wax.

3.2. N and micronutrient retention by WSB suspended in seawater

Patterns of N leaching for both beads types over a 2 h period indicated that there was an initial 5–10 min period of rapid N loss, followed by much slower N loss over the remaining 2 h of bead suspension. WSB prepared with 100% wax retained N significantly better than beads prepared with 95% wax/5% mPHL over the 2 h period (ANOVA, $P<0.0001$), with 100% wax beads retaining 44% of initial N compared with 24% N for beads prepared with 95% wax/5% mPHL (Fig. 1).

Micronutrient retention by WSB prepared with either 100% bees wax or 95% wax/5% mPHL after 4 min suspension in seawater is shown in Fig. 2. It was only possible to statistically compare retention efficiencies for vitamins and amino acids between these two WSB types because retentions of other nutrients were only measured in duplicate samples. Nonetheless, it was apparent that retention of micronutrients by 100% wax beads was generally greater than for beads containing 95% wax/5% mPHL, and this difference was often statistically significant (Tukey/Kramer; $P<0.05$). Retention of trace metals and iodine by 100% wax beads ranged from 52% (iodine) to 100% (zinc) and riboflavin and thiamin were retained with efficiencies of 83 and 59%, respectively, with the more soluble thiamin being lost at higher rates. Vitamins A and E were retained by both bead types with little loss; however, only 69 and 6% astaxanthin remained in beads prepared with 100% wax or 95% wax/5% mPHL, respectively. The amino acids taurine, arginine and methionine were retained at efficiencies of 48–55% by 100% wax beads and 19–23% by 95% wax/5% mPHL beads after 4 min suspension. These amino acid retention efficiencies were slightly lower than total N retention efficiencies shown in Fig. 1.

3.3. Lipid peroxidation products

Concentrations of thiobarbituric acid-reactive substances (TBARS) used as an indicator of lipid peroxidation, were significantly (Tukey/Kramer test; $P<0.05$) higher in pre-leached compared with post-leached beads for both 100% wax and 95% wax/5% mPHL WSB (Table 4). Concentrations of TBARS in pre-leached beads prepared with 95% wax/5% mPHL were highest at 528 nmol g^{-1}.

3.4. Utilization of 100% WSB by Artemia

The 100% wax beads containing OTC had a mean diameter (±1 S.D.) of 9.50±5.29 μm while 100% wax spray beads containing riboflavin had a mean diameter of 6.79±4.44 μm and, therefore, a high proportion of the beads were within the size range of particles reported to be ingested by *Artemia* nauplii (Dobbeleir et al., 1980; Makridis and Vadstein, 1999; Han et al., 2005). Examination of the digestive systems of *Artemia* fed on 100% wax beads containing riboflavin indicated that they could breakdown the beads and liberate riboflavin. Liberated riboflavin was evident as areas of diffuse, yellow-green fluorescence, in contrast to brighter, yellow-fluorescent bead fragments (Fig. 3).

Table 4
Concentrations of thiobarbituric acid-reactive substances (TBARS) in wax spray beads (WSB) prepared with either 100% bees wax or 95% bees wax and 5% marine phospholipid extract (mPHL)

Concentration of TBARS (nmol g^{-1})±1 S.D.			
100% WSB		95% wax/5% mPHL WSB	
Pre-leached	Post-leached	Pre-leached	Post-leached
89.2±9.1[b]	43.2±1.9[a]	527.9±40.7[c]	102.2±26.1[b]

WSB contained a micronutrient mix (Table 2) and were suspended with agitation for 4 h at 20–22 °C in a 0.5% *w/v* sodium dodecyl sulfate solution made up in seawater. Samples ($n=4$) were taken initially ($t=0$ h) and after 4 h. TBARS concentrations that are significantly different (Tukey/Kramer test; $P<0.05$) are labeled with different superscript letters.

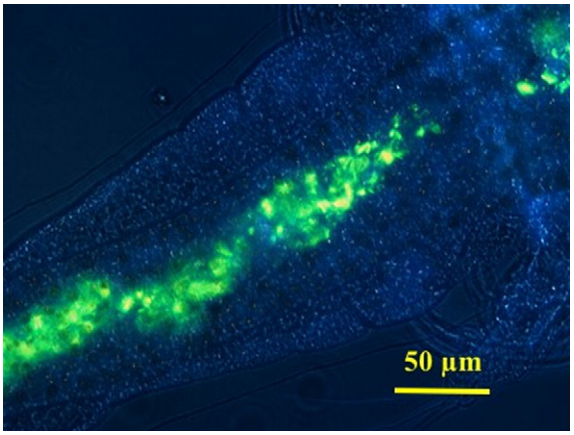

Fig. 3. Digestive system of *Artemia* fed on 100% wax beads containing riboflavin. Fragments of beads can be seen in the gut with diffuse fluorescence suggesting release of riboflavin. Samples were examined using a Leica DM1000 epifluorescent microscope (Leica Microsystems, Wetzlar, Germany; 40×/0.65 objective, excitation 450–490 nm, emission 515–528 nm) fitted with a Spot Insight QE color camera (Diagnostic Instruments, Sterling Heights, MI, USA).

The average OTC concentrations in *Artemia* were significantly different among treatments (two-way ANOVA, $P = 0.001$; Fig. 4); however, there was no overall difference ($P=0.1092$) between OTC concentrations in *Artemia* purged on algae and maintained in filtered seawater for 2 h after being fed on OTC-WSB. *Artemia* fed OTC-WSB retained 9.93±5.16 ng OTC (±1 S.D.) when purged with algae and 11.47±5.32 ng OTC when maintained in filtered seawater. In contrast, when immersed in an aqueous solution of OTC, only 0.24±0.42 ng OTC was retained by *Artemia* purged with algae and 2.85±2.64 ng OTC by *Artemia* maintained in filtered seawater. These latter OTC concentrations in *Artemia* were less (Tukey HSD; $P<0.05$) than concentrations obtained with OTC-WSB but not different (Tukey HSD; $P>0.05$) from those for *Artemia* exposed to gum arabic solution alone.

4. Discussion

The results of this study indicate that it is possible to prepare wax spray beads with high concentrations of non-labile micronutrients,

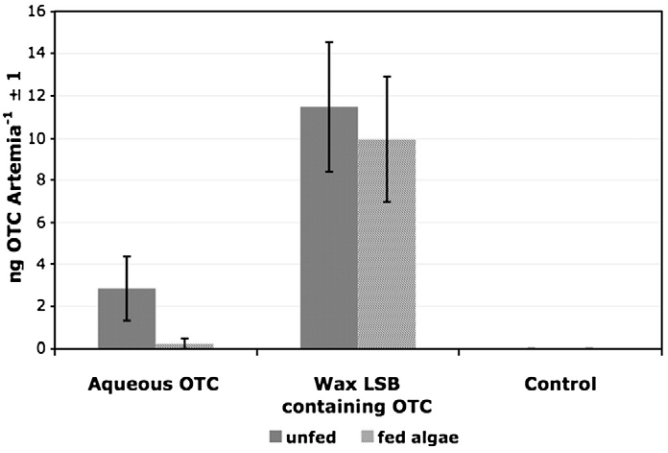

Fig. 4. Oxytetracycline uptake by *Artemia* after 22 h of enrichment followed by a 2 h purge period in clean seawater. *Artemia* were either fed WSB containing OTC or exposed to an equivalent amount of aqueous OTC in solution during enrichment. A third treatment consisted of *Artemia* unexposed to OTC. During the 2 h post-treatment purge period in clean seawater, half the *Artemia* from each treatment was fed algae to facilitate gut purging.

such as trace minerals and amino acids; however, losses of labile micronutrients were high during both preparation of the micronutrient mix and the beads. Both selenium and vitamin C were undetectable in pre-leached WSB; however, the apparent loss of vitamin C may have been due to problems in extracting the insoluble form of incorporated vitamin C (Stay-C 35) from wax beads for analysis, although this explanation would not account for the loss of selenium that required no extraction for analysis. Incorporation of soluble forms of ascorbate-2-phosphate should be evaluated in future studies. Concentrations of thiamin, vitamin A and E were reduced in pre-leached beads compared with expected concentrations. Astaxanthin did not appear to be lost in beads prepared with 100% wax but more than 84.5% was lost from beads prepared with addition of 5% mPHL.

Peroxidation of lipids occurred during bead preparation, with TBARS concentrations as high as 528 nmol g^{-1} for beads prepared with 5% mPHL. In contrast, pre-leached 100% bees wax beads had lower (Tukey/Kramer; $P<0.05$) TBARS concentrations of only 89 nmol g^{-1}. TBARS concentrations were lower in leached beads compared with pre-leached beads due to probable leakage of water-soluble TBARS products from the beads. High TBARS concentrations have also been reported in commercial lipid enrichment emulsions containing *n*-3 PUFA (Monroig et al., 2007b). The combination of high trace metal concentrations, *n*-3 PUFA, water and high temperatures during bead preparation likely contributed to lipid oxidation and loss of labile micronutrients (Dwivedi and Arnold, 1973; Sutton et al., 1996). Oxidative losses may be limited by addition of antioxidants and delivery of PUFA in separate particles (e.g. as lipid emulsions or microalgae) from those used for delivery of reactive micronutrients, such as trace metals.

Leakage patterns for 100% wax and 95% wax/5% mPHL beads were similar, with greatest loss of N occurring in the first 5–10 min of bead suspension followed by much lower and constant leakage rates over the remaining 2 h period. The rapid initial loss of micronutrients from WSB was likely due to dissolution of micronutrients either at or near the beads' surfaces (Önal and Langdon, 2004b). If the rate of N loss after the first 10 min period is assumed to be constant, the time period for 50% N loss (T_{50}) can be estimated to be 67 min for 100% wax beads versus <2 min for 95% wax/5% mPHL beads. These T_{50} values can be compared to values ranging from 3 to 90 min for glycine incorporated in WSB with different lipid compositions (Önal and Langdon, 2004b, 2005). It should be noted, however, that previous studies did not include the addition of dispersants when determining leakage rates of WSB and this could have resulted in clumping of suspended beads and consequent underestimates of leakage rates (Nordgreen et al., 2007).

Mixtures of micronutrients (minerals, water-soluble and fat-soluble vitamins) combined with amino acids, have not been previously incorporated in WSB. We found that WSB prepared with 100% bees wax showed higher micronutrient retention efficiencies, after a 4 min period of aqueous suspension, than beads prepared with 95% wax/5% mPHL and these differences were significant (Tukey/Kramer; $P<0.05$) for all tested water-soluble vitamins, amino acids, as well as astaxanthin. Retention efficiencies for riboflavin and amino acids in this study were comparable to those reported for spray beads prepared with other lipid types. For example, based on retention rate equations of Önal and Langdon (2004a), spray beads prepared methyl palmitate retained 94% riboflavin after 4-min aqueous suspension compared with an efficiency of 83% for 100% bees wax beads in this study. Önal and Langdon (2004b) reported that between 66 and 71% glycine (depending on the experiment) was retained by spray beads composed of 100% menhaden stearine after 4-min aqueous suspension whereas we found retention efficiencies varied from 48% (methionine) to 55% (taurine) for bees wax beads over the same time period. Even after losses due to oxidation and leakage, bead concentrations for most micronutrients were substantially higher than those reported in rotifers, *Artemia* and copepods.

Feeding experiments indicated that *Artemia* were capable of breaking down 100% bees wax beads and liberating riboflavin into their digestive systems. The mechanism of bead breakdown was not determined but it is likely that it was due to mechanical action of teeth and other cuticular projections of the mandibles that develop with the onset of feeding in stage 3 metanauplii (Tyson and Sullivan, 1981; Peterson and Rosowski, 1994). Digestion of wax esters by *Artemia* has not been studied extensively; however, several researchers have reported that *Artemia* nauplii can utilize ethyl esters of various fatty acids (Harel et al., 1999; Navarro et al., 1999), suggesting that limited enzymatic digestion of more complex waxes is possible. Limited enzymatic digestion of waxes and incorporation of resulting fatty acids by *Artemia* may be an advantage in that enrichments using WSB will less likely result in elevated concentrations of saturated fatty acids in these prey organisms.

Uptake of OTC by *Artemia* fed on OTC beads indicated that WSB successfully delivered water-soluble substances to *Artemia*. The use of wax beads to enrich *Artemia* with OTC could greatly reduce the required amount of OTC needed to obtain a desired concentration in fish larvae, compared with the traditional method of immersing larvae in an OTC solution.

Based on the results of this study, wax beads may be useful in improving the nutritional composition of *Artemia* and other live prey organisms for marine fish larvae. Many attempts have been made to enrich *Artemia* with nutritional supplements (see review by Dhont and Van Strappen, 2003), but biochemical processes can affect subsequent concentrations of nutrients in these organisms (Navarro et al., 1999; Chakraborty et al., 2007). Optimizing enrichment procedures using WSB is beyond the scope of the present study and will require further research.

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