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## NOTE

**MICROENCAPSULATION OF OKADAIC ACID AS A  
TOOL FOR STUDYING THE ACCUMULATION OF DSP  
TOXINS IN MUSSELS**

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Running title: Microencapsulation of okadaic acid.

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1 **ABSTRACT**

2           The possibility and effectiveness of microencapsulation of okadaic acid (OA) in  
3 gelatin acacia microcapsules has been studied. The encapsulation efficiency was *ca.*  
4 33%. The microcapsules were shown to be very stable, not leaching more than 9% of  
5 the encapsulated OA in a 20-hour period. OA from the microcapsules was absorbed by  
6 the mussels very efficiently, accumulating -after 3 days of feeding and one of  
7 depuration- 65% of the OA in microcapsules and 22% of the total OA used at the  
8 beginning of the microencapsulation process. These efficiencies and the possibility of  
9 encapsulating single DSP toxins and derivatives constitute a valuable tool for the study  
10 of the accumulation and biotransformation of DSP toxins in bivalves.

11

12 **Keywords:** Microcapsules, okadaic acid, absorption efficiency, biotransformation,  
13 mussels.

1 Diarrhetic shellfish poisoning (DSP) is a syndrome caused by some polyether  
2 compounds -okadaic acid (OA), analogs and derivatives- that produce diarrhoea,  
3 nausea, vomiting, headache, chills and moderate to severe abdominal pain in humans  
4 (Yasumoto et al., 1978). The responsible toxins are produced by several species of  
5 dinoflagellates belonging to the genus *Dinophysis* and *Prorocentrum* (Yasumoto et al.  
6 1980, Murakami et al., 1982; reviewed in Blanco et al., 2005) and are accumulated by  
7 shellfish that feed on these species. DSP toxicity probably has the highest impact on  
8 bivalve aquaculture, as it is frequently found in areas in which that activity is intense,  
9 such as Galicia (Spain) or southern Chile.

10 Mitigating the consequences of these blooms requires understanding the  
11 mechanisms involved in the absorption, elimination and transformation of this group of  
12 toxins by the bivalves. Experimental approaches are hampered by the difficulty of  
13 obtaining cultures of DSP-producing organisms and even when those are obtained, the  
14 cells usually contain a complex mixture of toxins and toxin derivatives that makes  
15 interpretation of the results difficult.

16 In order to supply the bivalves with food containing individual toxins or  
17 derivatives, we have studied the possibility and the effectiveness of microencapsulation  
18 of okadaic acid in gelatin-acacia microcapsules.

19 To obtain okadaic acid, naturally contaminated mussels were dissected, weighed  
20 and their digestive glands homogenized in methanol in a proportion of 1 g of tissue (4  
21 mL of extraction solvent), with an Ultraturrax T 25 Basic (IKA). The obtained  
22 homogenate was centrifuged at 48000 g for 20 min to clarify the solution (Beckman  
23 Avanti J-25). The extract was diluted to 50% methanol and subjected to liquid-liquid  
24 partitions with hexane and dichlorometane. The absence of conjugated forms (acyl  
25 derivatives or diol-esters) in the extract was checked by means of alkaline hydrolysis,

1 that transforms them to free OA. The dichloromethane fraction was evaporated to  
2 dryness and redissolved in 0.5 mL of olive oil. Aliquots of 0.25 mL were used to  
3 quantify OA in the extract and to be microencapsulated respectively

4 Gelatin-acacia microencapsulation was based on the complex coacervation method  
5 used by Planas et al. (1990) but with slight modifications (Figure 1). Once the  
6 microcapsules had been produced they were washed by allowing them to decant,  
7 removing supernatant water, adding new water and shaking the suspension gently. This  
8 operation was repeated each day during the experiment in order to avoid lump  
9 formation.

10 The encapsulation efficiency was quantified by the proportion of OA that was  
11 successfully encapsulated. OA contained in the microcapsules was quantified by  
12 extraction of *approx.* 1 g of microcapsules, with methanol 100% (1:4, v/v) and  
13 clarification by centrifugation for 20 min. at 48000 g.

14 The capability of the microcapsules to retain the toxins when they are suspended  
15 in seawater was also studied. Microcapsules were placed in seawater and samples of this  
16 water were taken at different times (0, 1, 2, 3 ,4 and 20 h). An initial blank of the  
17 seawater was also analysed. OA was extracted from the water by liquid-liquid partition  
18 with dichloromethane, evaporation of the organic fraction, and resuspension in 100%.  
19 methanol

20 Finally, we have shown that the toxins from microcapsules can be absorbed by  
21 mussels and they can be traced after being fed. OA-containing microcapsules were fed  
22 to two groups of six mussels obtained from the same batch which contained low levels  
23 of toxins (Table I). The mussels were placed in a beaker and were fed with a diet  
24 constituted by 10 mL of the microcapsules suspension (approximately 1 g of  
25 microcapsules) plus 25 mL of a culture of the microalgae *Tetraselmis* sp. ( $3 \times 10^6$

1 cels·mL<sup>-1</sup>) plus 0.05 g of clay. The food was supplied twice a day during 3 days. After  
2 the third day of feeding, mussels were maintained without food for an additional day, in  
3 order to remove most undigested food from the digestive system before analysis. This  
4 step was needed because very likely not all microcapsules are digested and, if they are  
5 not removed from the gut, the toxin they contain would be measured jointly with the  
6 absorbed one thus making it impossible to discriminate the absorbed from the ingested  
7 toxin. Furthermore, feces were collected from the bottom of the beaker every day and  
8 pooled the last day of the experiment. At the end, the digestive glands were  
9 homogenized in methanol (100%) in a proportion of 1:4, w:v, with an Ultraturrax T 25  
10 Basic (IKA) and the homogenate obtained was centrifuged at 48000 g for 20 min to  
11 clarify the solution. This extract was analyzed to quantify free OA. In order to quantify  
12 the total amount of toxin (OA and its conjugated forms (CF)) the methanolic extracts  
13 were subjected to an alkaline hydrolysis (after Vale and Sampayo, 1999, but using a  
14 temperature of 80°C). The extracts were desalted and partially cleaned with polymeric  
15 reverse-phase SPE cartridges Strata X (60 mg/3 mL) (Phenomenex). Feces were  
16 subjected to the same procedure, with the exception that the proportion of methanol  
17 added to extract the toxins was 4 mL of methanol per mL of feces, instead of per gram  
18 as in digestive glands.

19 OA concentration in each extract was determined by HPLC-ESI-MS with a  
20 Thermo LCQ-Deca XPplus ion trap mass spectrometer with electrospray ionization  
21 coupled to a Thermo Surveyor HPLC. The chromatographic separation was carried out  
22 using the method of Quilliam (2003) slightly modified to adapt it to a fused core column  
23 (HALO C8 50x2.1mm 2.7 µm). The two mobile phases were: A= H<sub>2</sub>O: F (95:5) and B=  
24 CH<sub>3</sub>CN: F (95:5), where F was 40 mM ammonium formate and 1M formic acid in  
25 water. The gradient program went from 5% to 100% B over 5 min, hold for 5 min and

1 then re-equilibrate at the starting conditions for 5 min prior to the next injection. Flow  
2 rate was  $400\mu\text{L min}^{-1}$  and injection volume  $5\mu\text{L}$ . The mass spectrometer was operated  
3 in negative ionization mode and SIM (selected ion monitoring) for  $803.5\ m/z$ .  
4 Quantification of the OA was carried out by comparing the resulting response with that  
5 of the reference materials (OA-1b) obtained from IMB - NRC, Canadá. The presence of  
6 matrix effect was checked by serial dilutions of the samples and corrected when needed  
7 by using the results obtained with the largest dilution.

8         Some modifications to the microencapsulation technique of Planas et al. (1990)  
9 were made in order to adapt the microcapsule size to the requirements of mussels. In  
10 this sense we have used a pH of 7 for the step of mixture of colloids and of 4.2 for the  
11 emulsification, obtaining microcapsules with a diameter about  $50\ \mu\text{m}$ . Also a  
12 homogenization with Ultraturrax at 24000 rpm (instead of the sonication of the original  
13 technique) was introduced to favor a correct mixture between the OA-olive oil and the  
14 acacia solution.

15         The efficiencies of encapsulation of the oils (olive and cod liver oil) were always  
16 high and similar to the ones obtained by Planas et al. (1990) encapsulating cod liver oil  
17 (50 to 85%) or by Knauer and Southgate (1997) encapsulating lipids (ca. 80%).  
18 However the encapsulating efficiency of OA was lower, ranging from 33 to 53%. This  
19 difference is probably due the partially hydrophilic nature of OA, (its partition  
20 coefficient between water and olive oil which is roughly 0.3, as we have estimated in a  
21 preliminary experiment in which ca. 30% of okadaic acid remained in water after a 1:1  
22 water:olive oil liquid-liquid partition).

23         The stability of the microcapsules in seawater was good, as the OA they contain  
24 did not leach substantially. Only about 8.5% of the toxin contained in the microcapsules  
25 was lost as soon as they were suspended in seawater (0 hours), probably due to an

1 insufficient washing, and no significant loss (c.a. 0.5%) could be detected after that  
2 time. Planas et al. (1990) found a similar leaching percentage for cod liver oil but during  
3 an 8-hour period notwithstanding, with no noticeable loss at 0 hours.

4       Mussels fed well on microcapsule suspensions. At the end of each 24h feeding  
5 cycle no suspension in the water was apparent and no pseudofeces detected, indicating  
6 that all the food supplied was ingested. At the end of the experiment the six mussels had  
7 increased their total toxin content (OA free + OA Conjugated forms) in the digestive  
8 gland (DG) by 1.857  $\mu\text{g}$  (Table 1). This accumulation by the mussels represents 63% of  
9 the microencapsulated toxin and 22% of the initial toxin amount. A large proportion of  
10 the toxin is therefore lost but enough of it was retained by mussel as to consider the  
11 technique useful to study toxin accumulation and bio-transformation. In feces, only a  
12 small percentage of free OA was found and most was found as conjugated forms. From  
13 this proportion of free OA an absorption efficiency higher than 88% could be estimated  
14 (assuming that the free okadaic acid is not transformed in the gut, and that consequently  
15 the free OA found in feces comes from the non-absorbed toxin plus the first absorbed  
16 and then excreted free toxin).

17       It can be concluded that microencapsulation is a viable way of supplying toxic  
18 food for studies of accumulation and biotransformation of biotoxins in bivalves.  
19 Mussels feed well on gelatin-acacia microcapsules if they are mixed with microalgae.  
20 The microencapsulated toxins can be readily absorbed allowing a substantial increase in  
21 toxin concentrations of the mussels in a few days.

22       It seems clear that this technique is more feasible for studying the  
23 biotransformation and the mechanisms involved in accumulation than using *Dinophysis*  
24 or *Prorocentrum* cultures and also than the study of naturally intoxicated populations.  
25 In all cases because the complexity of the toxin mixture could be controlled and

1 simplified. In the case of *Dinophysis* cultures, because the AO producing species in this  
2 genus have complex nutritional requirements. *D. acuminata*, for example, requires (as  
3 far as we know presently) a partially photosynthetic ciliate (*Myrionecta rubra*) as prey.  
4 The ciliate must obtain the chloroplasts from a cryptophyte (usually *Teleaulax*) (Park  
5 et al. 2006). Culturing this species requires consequently three steps (one for each  
6 species) that should be carefully synchronized. That is an expensive and time  
7 consuming process, which is additionally prone to fail due to contamination of the final  
8 *Dinophysis* culture with the cryptophytes. In relation to *Prorocentrum*, using  
9 microcapsules has advantages because the species in this genus are inefficiently  
10 digested by mussels, and (and perhaps because of), at least some of them produce a  
11 number of bioactive compounds (Cruz et al., 2008; Napolitano et al., 2009; Napolitano  
12 et al., 2010). Microcapsules can be used to supply mussels with individual DSP toxins  
13 derivatives that have lower polarity than okadaic acid, such as 7-O-acyl derivatives or  
14 diol esters, but its use with more polar derivatives, such as “DTX4” and “DTX5”,  
15 should be additionally evaluated.

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9

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1

2

ACCEPTED MANUSCRIPT

1 **FIGURE AND TABLE CAPTIONS**

2

3 **Figure 1:** Schematic diagram of the preparation of okadaic acid gelatin-acacia  
4 coacervate microcapsules (Partially from Planas et al., 1990).

5

6 **Table 1:** Content of okadaic acid (OA) and their conjugated forms (CF) in the digestive  
7 gland of mussels and feces at the beginning and the end of the experiment.

8 DG = digestive gland; Day 1 = the first day that the mussels were fed. Day 4 = the day  
9 after the last feeding.

10

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0.5 g of Acacia dissolved in 50 mL of water 40°C

0.5 g of Gelatin dissolved in 50 mL of water 40°C

Mix

0.25 mL dichloromethane (okadaic acid) fraction redissolved in olive oil.

Homogenization with Ultraturrax 24000 rpm 20 sec.

Adjust pH to 7 with NaOH 0.1N

Adjust pH to 7 with NaOH 0.1N

Emulsify

(Magnetic stirrer)  
Stirring rate: 250 rpm.  
30 sec.

Adjust pH to 4.2 with HCl 0.1N.  
Stirring rate: 250 rpm

Gelation of coacervates by cooling in an ice bath for 20 minutes.  
Stirring rate: 100 rpm

Adjust pH to 9.0 with NaOH 0.1N  
Agitate for 30 minutes  
Stirring rate: 100 rpm

Let stand the mixture for 2 hours at 4°C

	Type of Toxin	Toxin in Day 1 (T0) (µg)	Toxin Day 4 (TF) (µg)	Toxin increase (µg)
<b>Mussels</b>	OA	0.4750	0.7802	0.3052
	CF+OA	1.0973	2.9545	1.8572
<b>Feces</b>	OA	Not sampled	0.1663	-
	CF+OA	Not sampled	1.3706	-