

# VITELLOGENIN-LIKE PROTEINS IN CLAMS AND NONYLPHENOL LEVELS IN WATER, SEDIMENTS AND BIOTA: THE LAGOON OF VENICE AS A CASE STUDY

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## Introduction

Nonylphenol (NP) is commonly used in the production of nonylphenol ethoxylates (NPEs), NP phosphites and insecticides. As a consequence of their widespread use, particularly of NPEs as nonionic surfactants in household and industrial detergents, large quantities of these compounds are discharged into aquatic environments, either directly from untreated effluent or indirectly from sewage treatment plants (STPs). In aquatic ecosystems, NPEs biodegrade to de-ethoxylated intermediates, whose final product is NP. It is known that NP can have estrogenic effects in aquatic organisms, being able to mimic the action of endogenous estrogens by binding to estrogen receptors. As a consequence, it can induce vitellogenin (Vg) synthesis, the major precursor of the egg-yolk proteins of oviparous organisms.

## Aims

In this study, Vg induction was evaluated in the clam *Tapes philippinarum* (Fig. 1A) seasonally collected (Fig. 1B-D) from October 2003 to June 2004 in 3 sites of the Lagoon of Venice: Marghera (MA), characterised by high industrial contamination levels, Campalto (CA), near a STP, and Poveglia (PO) close to the lagoon inlet, which was considered as a reference site (Fig. 2).

In addition, NP and NPEs levels were evaluated in clam, water and sediment samples.

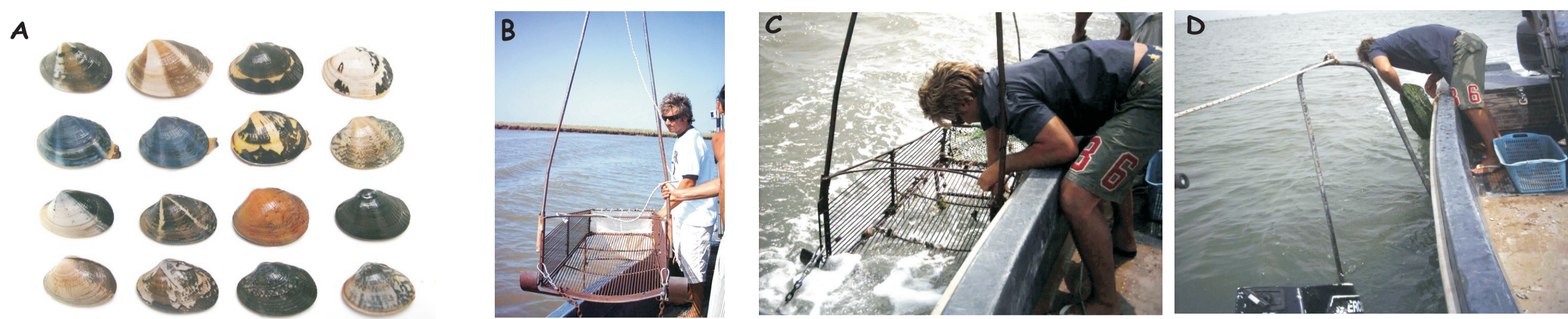


Fig. 1 - A) Specimens of *Tapes philippinarum*; B-D) phases of clam sampling in the Lagoon of Venice.

Extraction conditions	Tab. 1
<b>Water</b>	1L sample was extracted at 10 mL/min on Solid Phase Strata C18 (500 mg, 6 mL Phenomenex, USA) cartridge, eluted with acetone and concentrated under nitrogen stream.
<b>Sediment</b>	5 g extracted on automated Randall system with 120 mL methanol at reflux (immersion step: 2 h and washing: 3 h). Clean-up on Silica gel.

Analytical conditions	Tab. 2
<b>Chromatographic column</b>	Luna hexyl-phenyl (150 x 4.6 mm, 4 μm, Phenomenex)
<b>Mobile Phase</b>	Water:Methanol:linear gradient from 40:60 to 20:80 followed by 10 min isocratic elution at 20:80
<b>Flow rate</b>	1 mL/min
<b>Detector</b>	Fluorimetry, $\lambda_{exc}$ = 230 nm, $\lambda_{em}$ = 302 nm
<b>Injection volume</b>	20 μL

## Material and Methods

Vg-like proteins were determined by the alkali-labile phosphate (ALP) method (Blaise et al., Environ. Toxicol. 14: 455-465, 1999) in both the haemolymph and digestive gland of sexually differentiated males and females when it was possible to distinguish them by microscopic observation of a smear of gonadal tissue (Fig. 3). In the haemolymph  $Ca^{2+}$  levels were also measured, this parameter being strictly related to the presence of Vg-like proteins.

During clam samplings, water and sediments were also collected (Fig. 4) to determine the concentrations of NP (Tab. 1, 2), which was chosen as a marker of the presence of anthropic sources of estrogenic compounds.

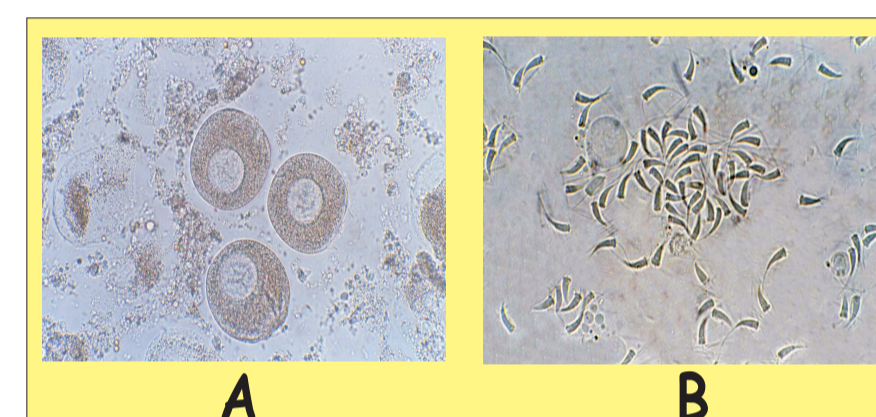


Fig. 3 - Oocytes (A) and spermatozoa (B) of *T. philippinarum*.



Fig. 4 - Sediment sampling in the Lagoon of Venice.

## Results...

Both male and female clams collected at CA and MA generally showed significantly higher Vg-like proteins in haemolymph with respect to specimens from Poveglia throughout the study period (Fig. 5A, 5B). Significant differences in Vg-like proteins were also detected in digestive gland of clams from the 3 sampling sites (Fig. 6A, 6B). In particular, sexually undifferentiated clams collected at CA showed the highest protein levels in January 2004, suggesting that endocrine alterations mostly occurred far from the reproductive period of animals.  $Ca^{2+}$  levels were generally higher in haemolymph of both female and males clams from CA and MA.  $Ca^{2+}$  levels showed also a clear seasonal trend, with higher values in spring-summer period than in autumn-winter (Fig. 7A, 7B).

Among sampling sites, MA and CA showed higher sediment NP concentrations than PO. The highest NP concentrations, ranging from 64 to 246 μg/g d.w., were always detected in sediment from MA, whereas the lowest NP concentrations, ranging from 27 to 98 μg/g d.w., were found in sediment of PO (Tab. 3).

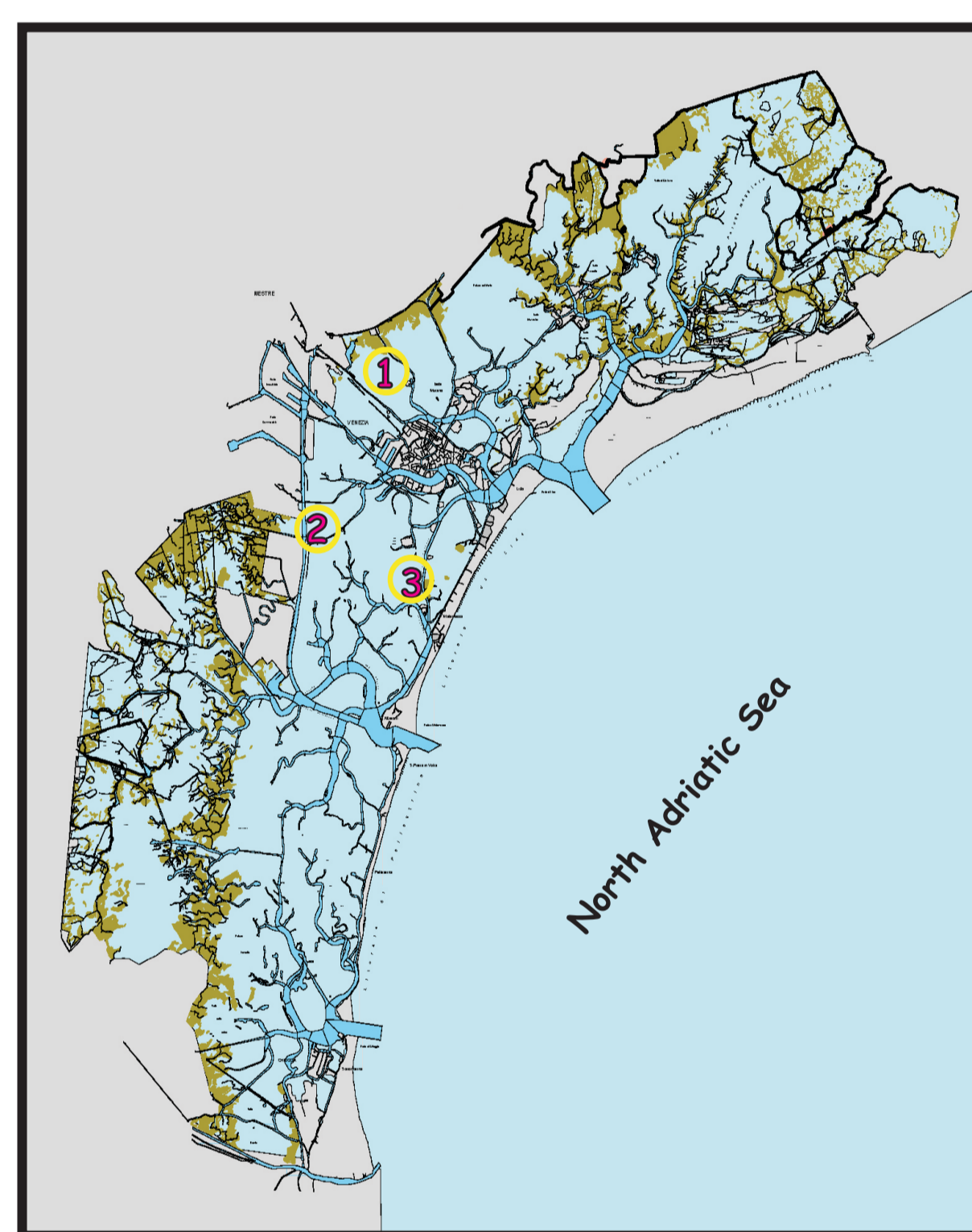


Fig. 2 - Sampling sites in the Lagoon of Venice. 1) Campalto; 2) Marghera; 3) Poveglia

Concentrations of nonylphenolic compounds (ng/g d.w.) in sediments from the Lagoon of Venice			
	NP	NPE	NP+NPE
<b>October '03</b>			
POVEGLIA	37,6 ± 9,9	30,2 ± 13,0	67,8 ± 22,4
CAMPALTO	45,1 ± 16,5	37,9 ± 7,8	83,0 ± 22,6
MARGHERA	64,5 ± 13,8	75,2 ± 15,4	139,6 ± 17,2
<b>January '04</b>			
POVEGLIA	98,6 ± 45,4	94,1 ± 54,7	202,4 ± 114,4
CAMPALTO	107,9 ± 52,3	144,4 ± 34,4	251,8 ± 86,7
MARGHERA	246,6 ± 0,0	304,1 ± 0,0	550,7 ± 0,0
<b>April '04</b>			
POVEGLIA	27,5 ± 8,7	44,9 ± 22,3	74,0 ± 33,4
CAMPALTO	106,0 ± 22,8	185,0 ± 86,3	291,1 ± 98,9
MARGHERA	223,6 ± 52,1	336,0 ± 73,9	559,6 ± 101,2
<b>June '04</b>			
POVEGLIA	51,8 ± 3,1	46,8 ± 68,2	98,6 ± 66,9
CAMPALTO	90,9 ± 2,9	123,7 ± 38,1	214,6 ± 35,5
MARGHERA	225,4 ± 27,6	318,7 ± 48,0	544,1 ± 89,9

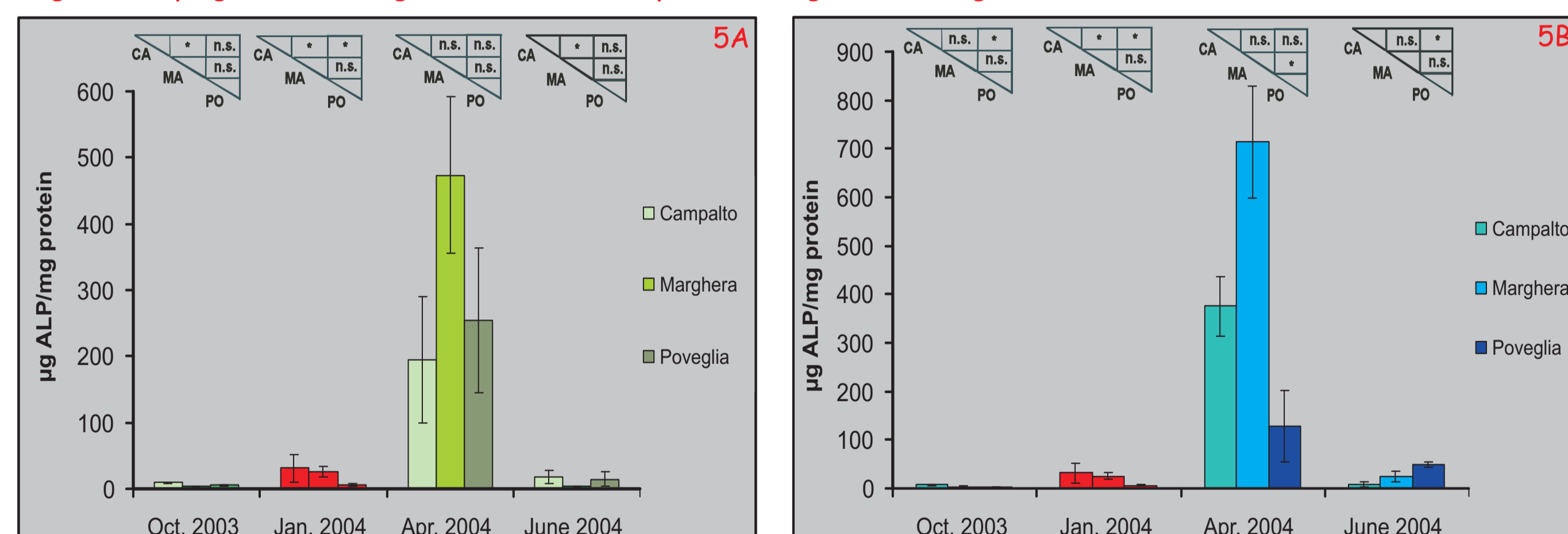


Fig. 5 - Vg-like protein levels (expressed as μg ALP/mg protein) in the haemolymph of females (A) and males (B) of *T. philippinarum*. Values are mean ± e.s.; n.s.: not significant, \* p<0.05. Red bars indicate Vg values in sexually undifferentiated clams.

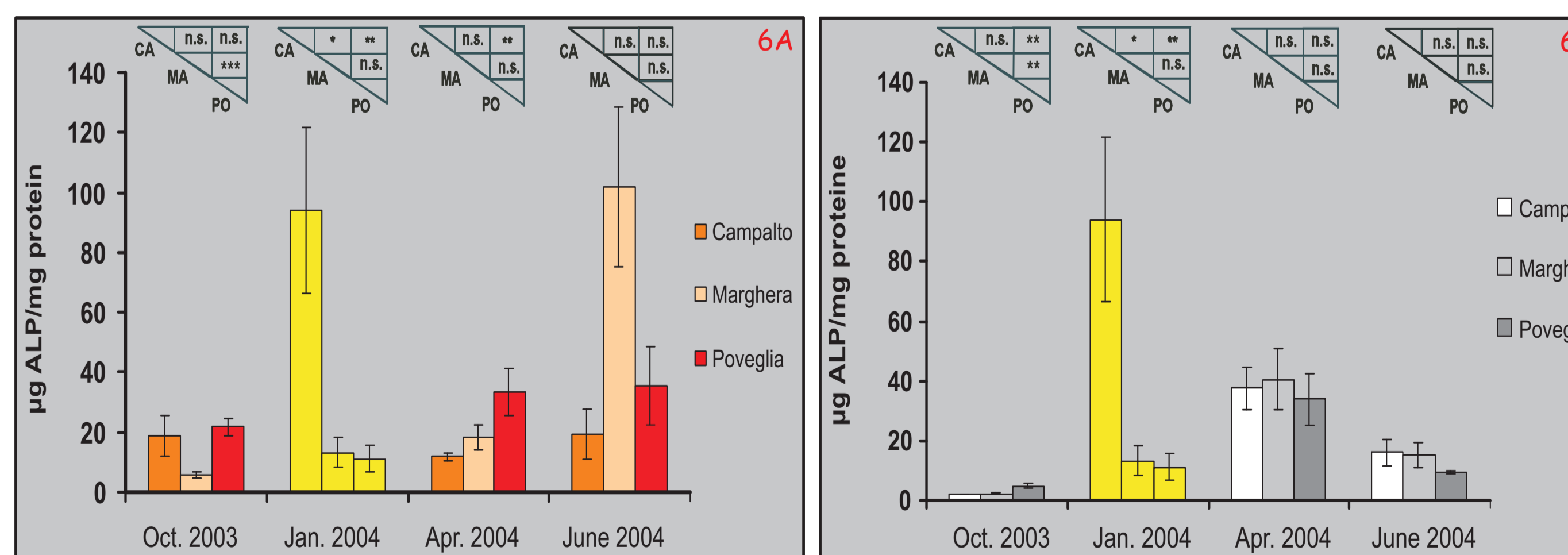


Fig. 6 - Vg-like protein levels (expressed as μg ALP/mg protein) in digestive gland of females (A) and males (B) of *T. philippinarum*. Values are mean ± e.s.; n.s.: not significant, \* p<0.05, \*\* p<0.01, \*\*\* p<0.001. Yellow bars indicate Vg values in sexually undifferentiated clams.

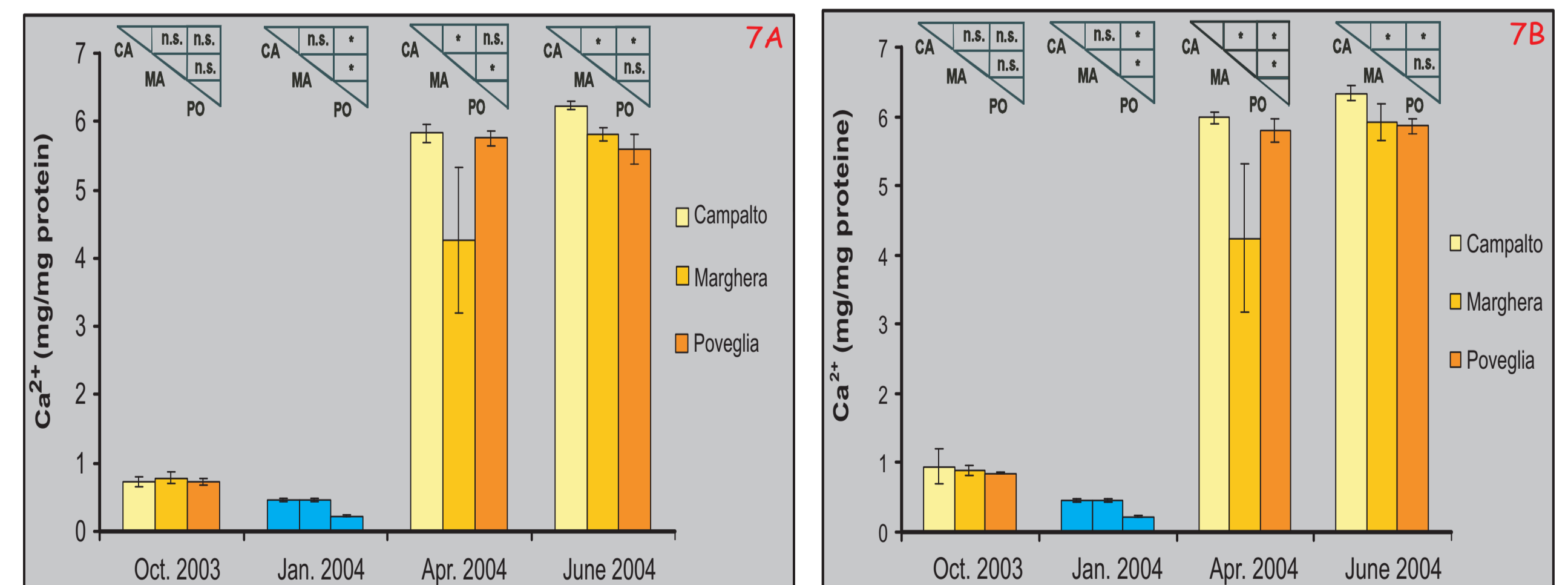


Fig. 7 -  $Ca^{2+}$  levels in the haemolymph of females (A) and males (B) of *T. philippinarum*. Values are mean ± e.s.; n.s.: not significant, \* p<0.05. Blue bars indicate  $Ca^{2+}$  values in sexually undifferentiated clams.

## ...Results

NP water concentrations were generally close to the analytical detection limit (50 ng/l) at all sampling sites.

The concentrations of NP in the range of 60-180 ng/g d.w. measured in clams, were instead of the same order of magnitude in the three sampling sites, the highest values were found in January and April in two sites (PO and MA) whereas the lowest value was measured in June in samples collected at CA. The concentrations of NPE1 and NPE2 were always below the limit of detection, thus reflecting on one hand the different lipophilic power of the compounds, on the other hand their water concentrations.

## Conclusions

The analyses on *T. philippinarum* showed a seasonal trend characterised by a raising in the NP concentration measured in soft tissues of clams collected from October to April, followed by a prompt decrease in June, when the sexual maturity and spawning phase are reached. In addition, this study demonstrates that Vg-like protein levels significantly increased in both hemolymph and digestive gland of sexually differentiated and undifferentiated clams from the most NP contaminated sites. Considering that endocrine disruption due to exposure to estrogenic compounds, such as NP, may cause fertility reduction, alteration in sex ratio, and a decrease in reproductive rate, a condition of potential risk for clam populations in estuarine areas is highlighted.