PRESENTATION N° I

THE EFFECTS OF DIATOM METABOLITES ON THE REPRODUCTIVE BIOLOGY OF COPEPODS

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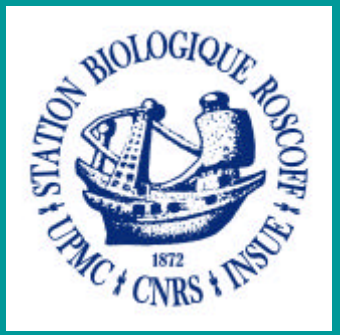


Background

Since the late 1950s, it was assumed that nutrient limitation, competition, predation and abiotic facto
etermine **demographic fluctuations** of plankton species over the year. Till now most plankton models are bas
n this presumption. Thus, food availability and food quality are generally assumed to be **key limiting factors** fo
quatic herbivores.

The descriptions of chemical interactions in plankton were mainly focused on few **metabolite marke**
f food quality, such as **polyunsaturated fatty acids**, that are assumed to be limiting the herbivore
eproduction success. The action of **phytoplankton toxins** arising mainly during dinoflagellates or
yanobacteria blooms has also been addressed with strongly controversial results. Only recently, evidence
ccumulated that species interactions during the average situation in the plankton are also regulated by a **"wate**
rms race" mediated by chemical or mechanical defenses. Since the majority of the involved **infochemicals** ar
ther **very dilute** in the open water or only **released during direct contact** of the protagonists, most of the
ctive principles are still awaiting discovery. Few examples of the direct action of chemical defense
nclude the influence of diatom-derived polyunsaturated aldehydes on copepod embryo hatching and
arval development and the role of dimethylsulfide as feeding deterrent.

The understanding of the **role of infochemicals** in the plankton is a key for an in-depth insight into t
teractions between primary producers and herbivores. Diverse expertise from modellers over geneticists to
cologists and chemists will be requested in the future to explore the action of infochemicals, which could explai
everal **open unexplored problems in plankton research**.

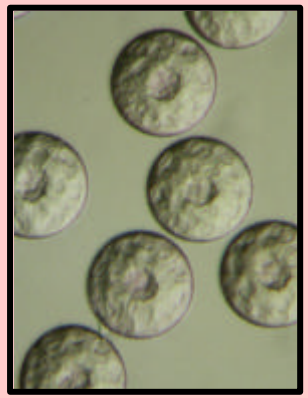
Innovative approaches are needed that allow studying **chemical fluctuations** in field populations as
ell as **chemical defence** of single species and communities. Since the concept introducing infochemicals as a
ew regulating aspect challenges the established view of plankton ecology it is currently under **controversial**
iscussion. Existing studies on the role of infochemicals are often criticized to focus on one or only few
arameters in laboratory experiments thus, not reflecting the complex field situation in the plankton. In contrast,
ost traditional approaches to understand processes that influence diversity are based on monitoring and
odeling, two techniques that allow describing and to certain extend predicting the annual species succession in
eir natural environment. It is due to this **methodological discrepancy** that despite growing evidence,
fochemicals are rarely discussed within the general framework of ecologically intact plankton. **Proven effects**
re often considered rather as exceptions in an environment which is otherwise exclusively shaped by

ZOOPLANKTON MODEL: *Calanus helgolandicus* **(Calanoid Copepod)**

Male + Female

Female

Eggs +Larva



Disadvantages

-Cultivation unknown
-Size of organisms
-High individual female heterogeneity
-Asynchron egg production
-Genome unknown

Advantages

-Transparent organism
-No remating
-Free spawner, subitaneous eggs
-Long spawning period
-Excellent representative

http://copepodes.obs-banyuls.fr/en/listegen.php

14 species

Calanus helgolandicus	(NA)
C. finmarchicus	(NA)
C. agulhensis	(SA)
C. pacificus	(NP)
C. sinicus	(NP)
C. chilensis	(SP)
C. glacialis	(AO)
C. australis	(AnO)

Other herbivorous/omnivorous copepods

Key category

Temora sp.	*Acartia sp.*	*Centropages sp.*	*Paracalanus sp.*	*Oithona sp.*
				
(¤),(+)	**(¤),(+)**	**(¤),(+)**	**(¤) ,(+)**	**(**),($)**

Small and very numerous – Key players in the **MFW**
(¤) Free spawner
(**) Egg-sac carrier (+ ***Pseudocalanus sp***.)
(+) Omnivorous (diatoms + other)
($) Diet linked to the Microbial Food Web

Studies of FEMALE versus MALE copepod reproduction

The descriptions of phytoplankton diet interactions with reproduction are mainly focused on FEMALE responses

The interactions are generally unknown for MALES

Descriptions are scarce, related to dinoflagellates **(1, 2),** or to insecticide **(3)**, not related to diatoms.

References-

(1)-Ianora, I., A. Miralto, I. Butino, S.A. Poulet and G. Romano. 1998. First evidence of some dinoflagellates reducing male copepod fertilisation capacity. **Limnology and Oceanography.** 44: 147- 153.

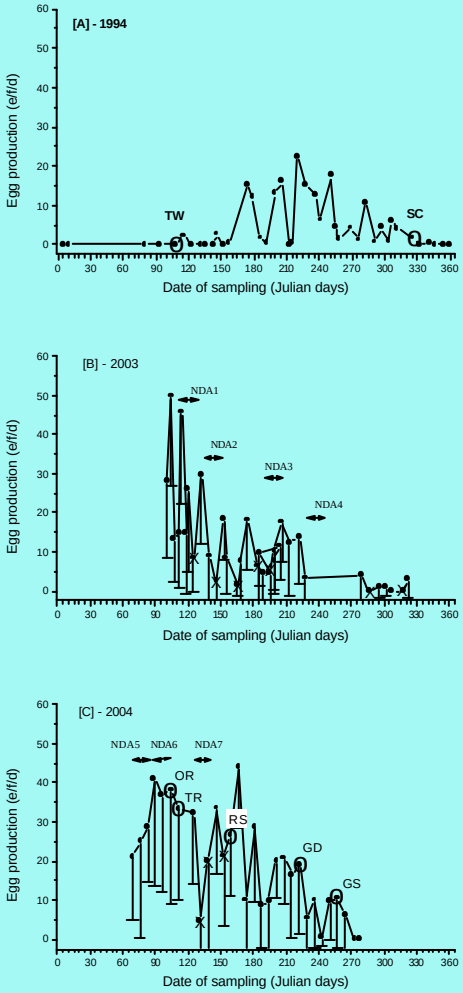
(2)-Laabir, M., Buttino, I., Ianora, I., Kattner, G., Poulet, S.A., Romano, G., Carotenuto, Y., and A. Miralto. 2001. Effect of specific dinoflagellate and diatom diets on gamete ultrastructure and fatty acid profile of the copepod *Temora stylifera*. **Marine Biology.** 138: 1241-1250.

(3)- Chandler-GT; Cary-TL; Volz-DC; Walse-SS; Ferry-JL; Klosterhaus-SL. 2004. Fipronil effects on estuarine copepod (*Amphiascus tenuiremis*) development, fertility, and reproduction: A rapid life-cycle assay in 96-well microplate format. **Envir. Toxic. Chem**. 23: 117-124.

State of the Art

I- The effects of algal diets impair different reproductive factors

Factor N° 1- EPR [Egg production rate. Unit: e/f/d]



FIELD

LABORATORY

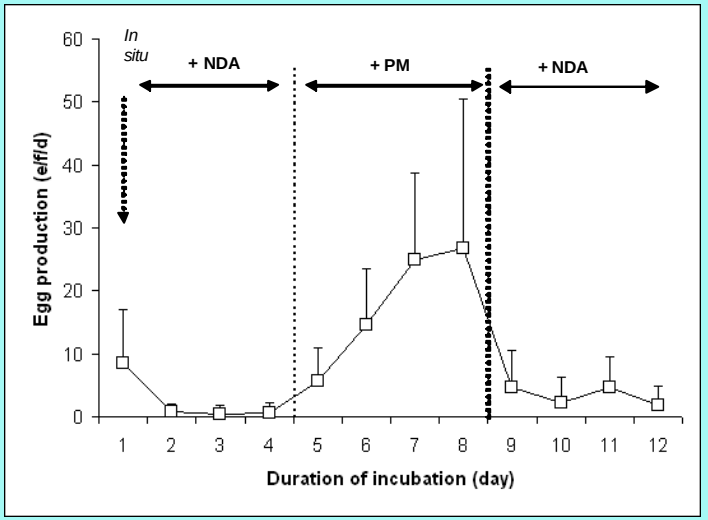


Figure 6 (new - REVISED)

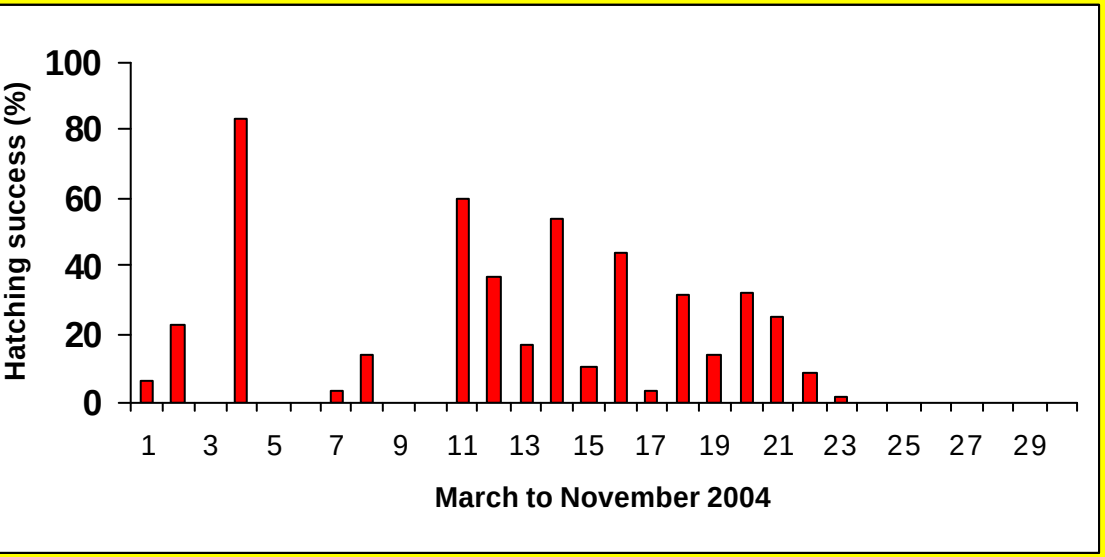
Reference-

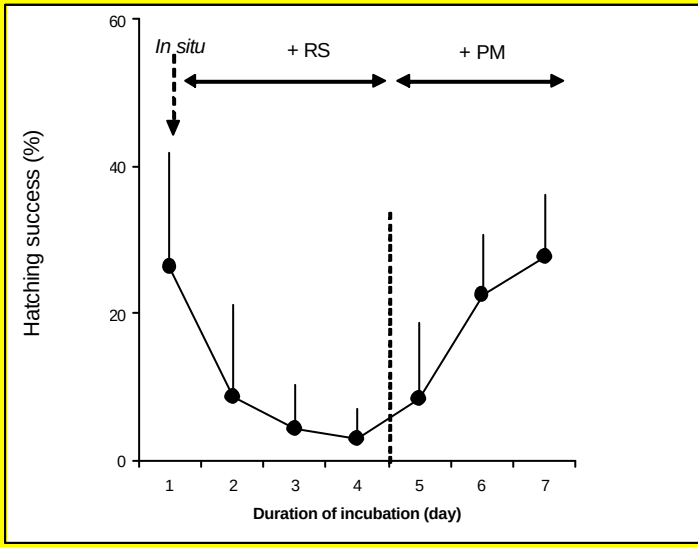
Poulet S. A., Wichard T., Ledoux J. B., Lebreton1 B., Marchetti1 J., Dancie C., Bonnet D., Cueff A., Morin P, Pohnert G. 2006. The influence of diatoms on copepod reproduction. **I.** Field and laboratory observations related to *Calanus helgolandicus* egg production. **Marine Ecology Progress Series**. 308: 129-142.

State of the Art

I- The effects of algal diets impair different reproductive factors

Factor N° 2- HS [Hatching success. Unit: %]





FIELD **LABORATORY**

Reference-

Wichard, T., Poulet S. A., Boulesteix A.L., Ledoux J.B., Lebreton B., Marchetti J., Pohnert G (2006) Influence of diatoms on copepod reproduction: **II.** The impact of diatom-derived a, ß, d, unsaturated aldehydes on the reproductive success of *Calanus helgolandicus* in coastal waters off Roscoff (Western English Channel, France). **Limnology & Oceanography** (submitted).

Poulet, S.A., Cueff, A., Wichard, T., Marchetti, J., Dancie, C., Pohnert, G (2006) Influence of diatoms on copepod reproduction. **III**. Consequences of abnormal oocyte maturation on three reproductive factors in *Calanus helgolandicus.* **Marine Biology** (submitted).

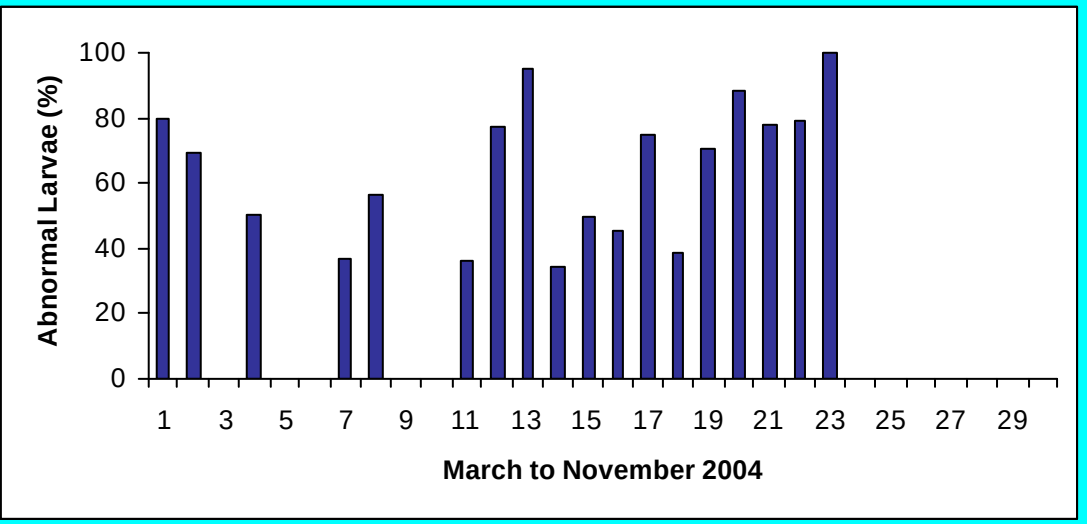
These eggs will hatch

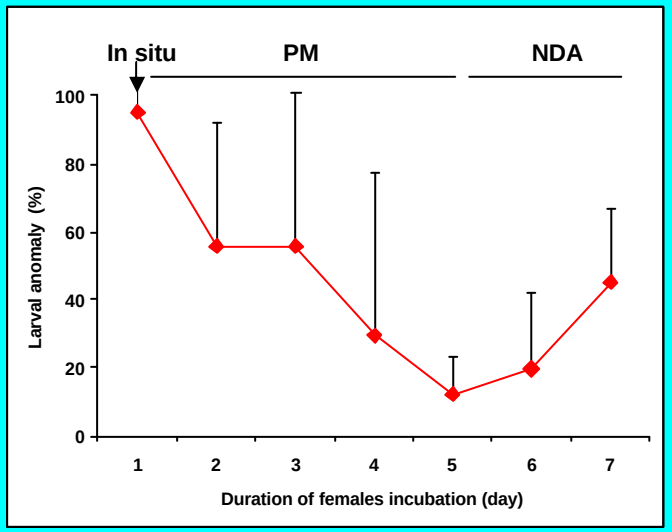
These eggs will not hatch

State of the Art

I- The effects of algal diets impair different reproductive factors

Factor N° 3- AL [Abnormal larva production. Unit: %]





FIELD **LABORATORY**

Reference-

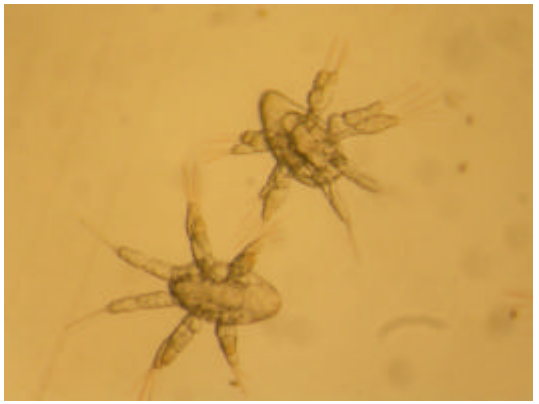
Wichard, T., Poulet S. A., Boulesteix A.L., Ledoux J.B., Lebreton B., Marchetti J., Pohnert G (2006) Influence of diatoms on copepod reproduction: **II.** The impact of diatom-derived a, ß, d, unsaturated aldehydes on the reproductive success of *Calanus helgolandicus* in coastal waters off Roscoff (Western English Channel, France). **Limnology & Oceanography** (submitted).

S.A. Poulet, A. Cueff, T. Wichard, J. Marchetti, C. Dancie, G. Pohnert; 2006. Influence of diatoms on copepod reproduction. **III**. Consequences of abnormal oocyte maturation on three reproductive factors in *Calanus helgolandicus.* **Marine Biology**. (submitted).

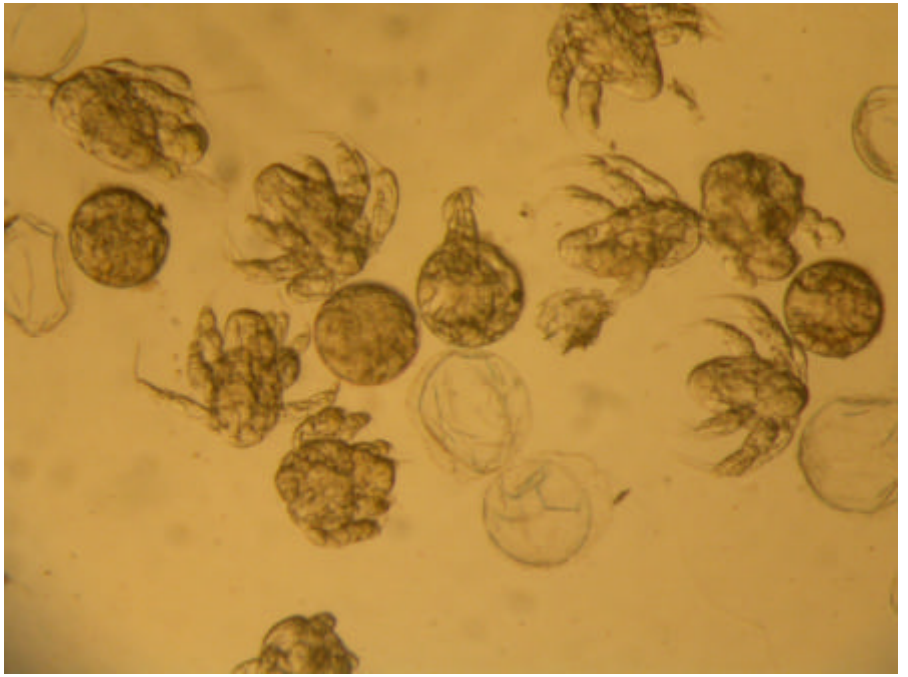
State of the Art

I- The effects of algal diets impair different reproductive factors

Symptom N°1- Morphological anomalies in larva



Normal Nauplii



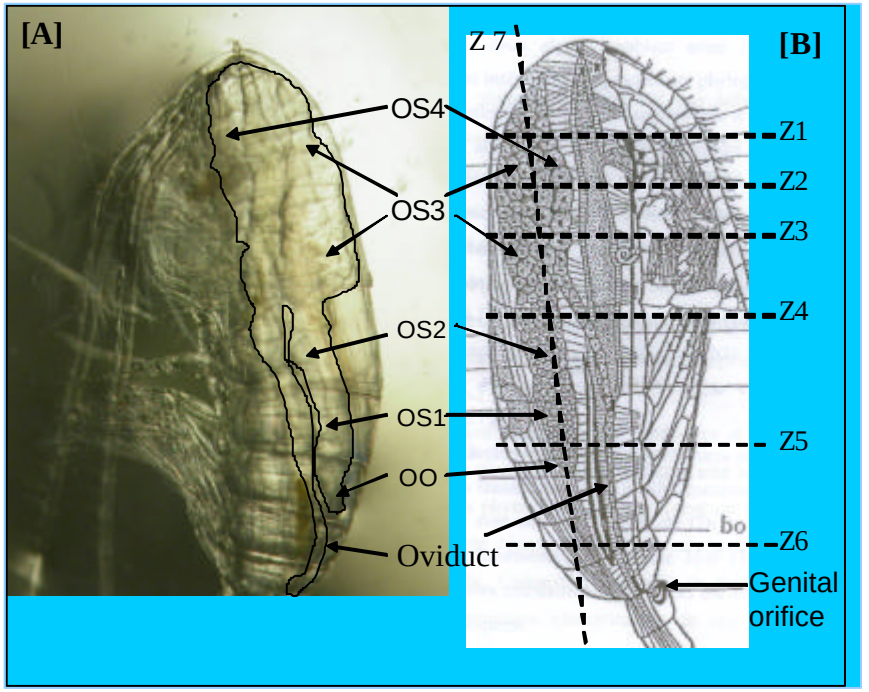
Abnormal Nauplii

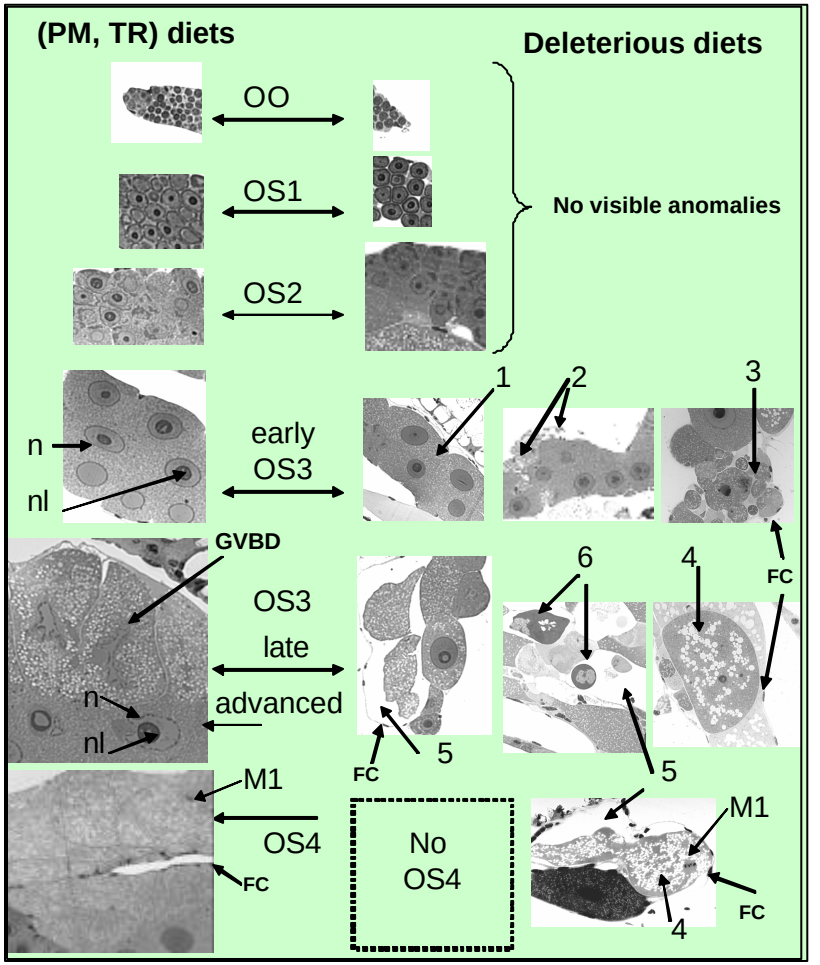
Reference-
Poulet, S.A., M. Laabir, A. Ianora and A. Miralto. 1995. Reproductive response of *Calanus helgolandicus*. I. Abnormal embryonic and naupliar development. **Marine Ecology Progress series**. 129 : 85-95**.**

State of the Art

I- The effects of algal diets impair different reproductive factor

Symptom N° 2- Cytological anomalies in gonads





Reference-
S.A. Poulet, A. Cueff, T. Wichard, J. Marchetti, C. Dancie, G. Pohnert; 2006. Influence of diatoms on copepod reproduction. **III**. Consequences of abnormal oocyte maturation on three reproductive factors in *Calanus helgolandicus*. **Marine Biology**. (submitted).
See also Neihoff 's publications for other copepods.

State of the Art

I- The effects of algal diets impair different reproductive factors

Symptom N°3- Cell anomalies (female oocytes and larva)

TEM observation of Oocytes
Atresia
Blebbing
Apoptotic body

Epifluorescent observation of Nauplii
Apoptosis

Reference-

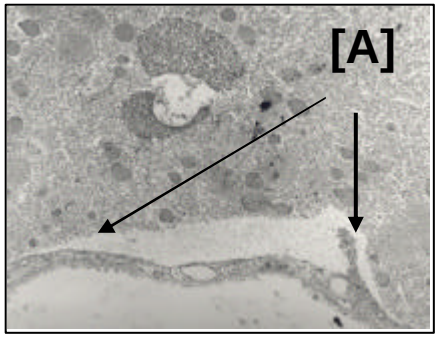
Poulet S.A., Richer de Forge, M., Cueff A., and J-F. Lennon. 2003. Double labelling methods used to diagnose apoptotic and necrotic cell degradations in copepod nauplii. Marine Biology. 143 : 889-895.

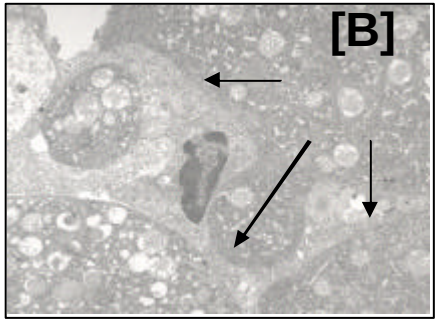
Buttino-I; Santo-MD; Ianora-A; Miralto. 2004. A Rapid assessment of copepod (*Calanus helgolandicus*) embryo viability using fluorescent probes. Marine Biology. 145: 393-399

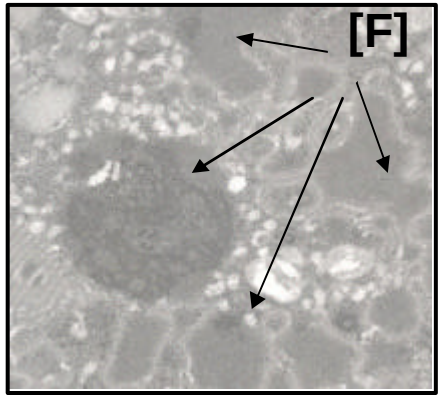
Adolph S, Bach S, Blondel M, Cueff A, Moreau M, Pohnert G, Poulet SA, Wichard T, Zuccaro A. 2004. Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla. Journal of Experimental Biology. 207: 2935-2946

Poulet S.A, Cueff A. 2006. TEM investigation of apoptotic cell anomalies in copepod oocytes mediated by diatoms. (in prep.).

TEM observation of Oocytes
Atresia **[A]**
Blebbing **[B]**
Fragmented body **[F]**

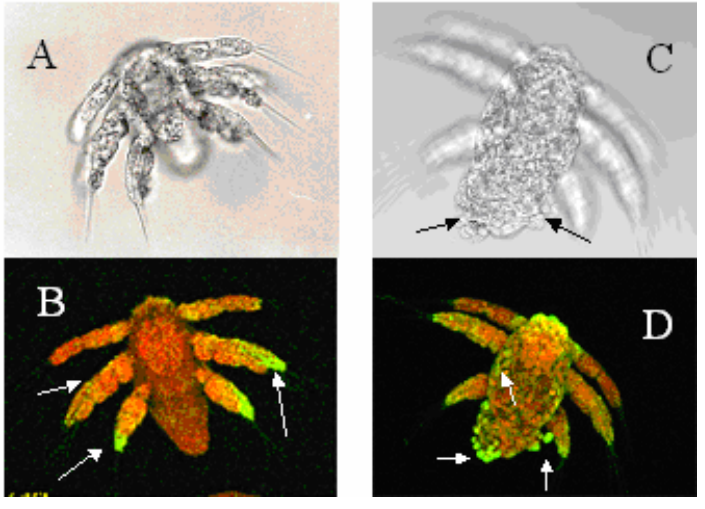






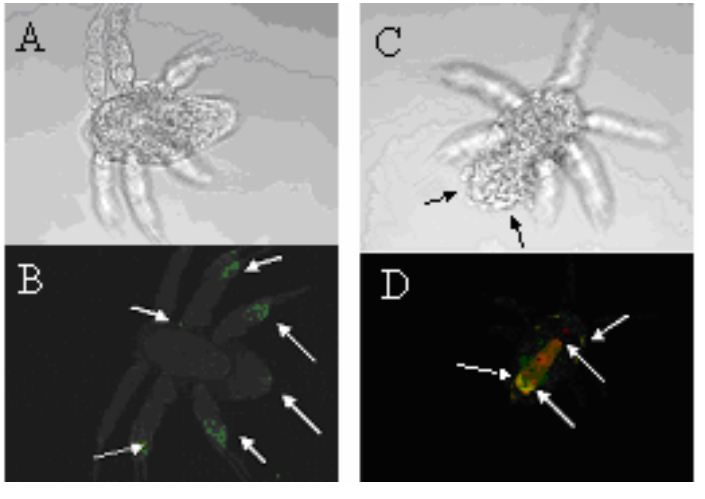
Epifluorescent observation of Nauplii
(TUNEL+propidium iodide and ANNEXIN V-FITC+propidium iodide)

[T+ Pi] →



Apoptotic symptoms

[A+Fi+Pi] →



II- The phytoplankton diets : DIATOMS

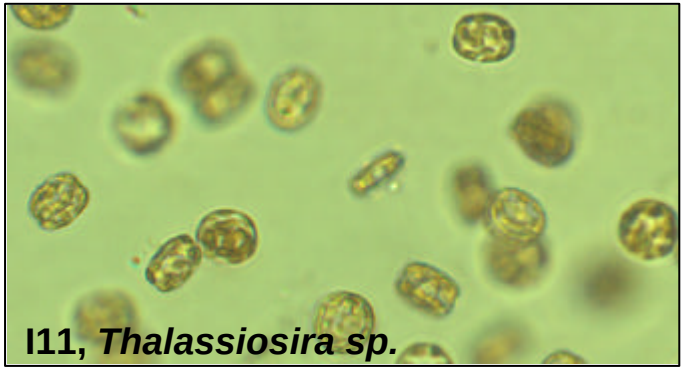
I1, *Thalassiosira sp.*

I2, *Odontella regina*

I4, *Dityllum breightwellii*

I6, *Thalassiosira rotula*

I8, *Thalassiosira sp.*



I11, *Thalassiosira sp.*

The four categories of effects (Reference N° 1)

1- EPR +; HS + (no inhibition)

2- EPR - ; HS +
3- EPR +; HS –
4- EPR - ; HS - } (inhibition)

Same categories of effects with native species **(Reference N° 2)**

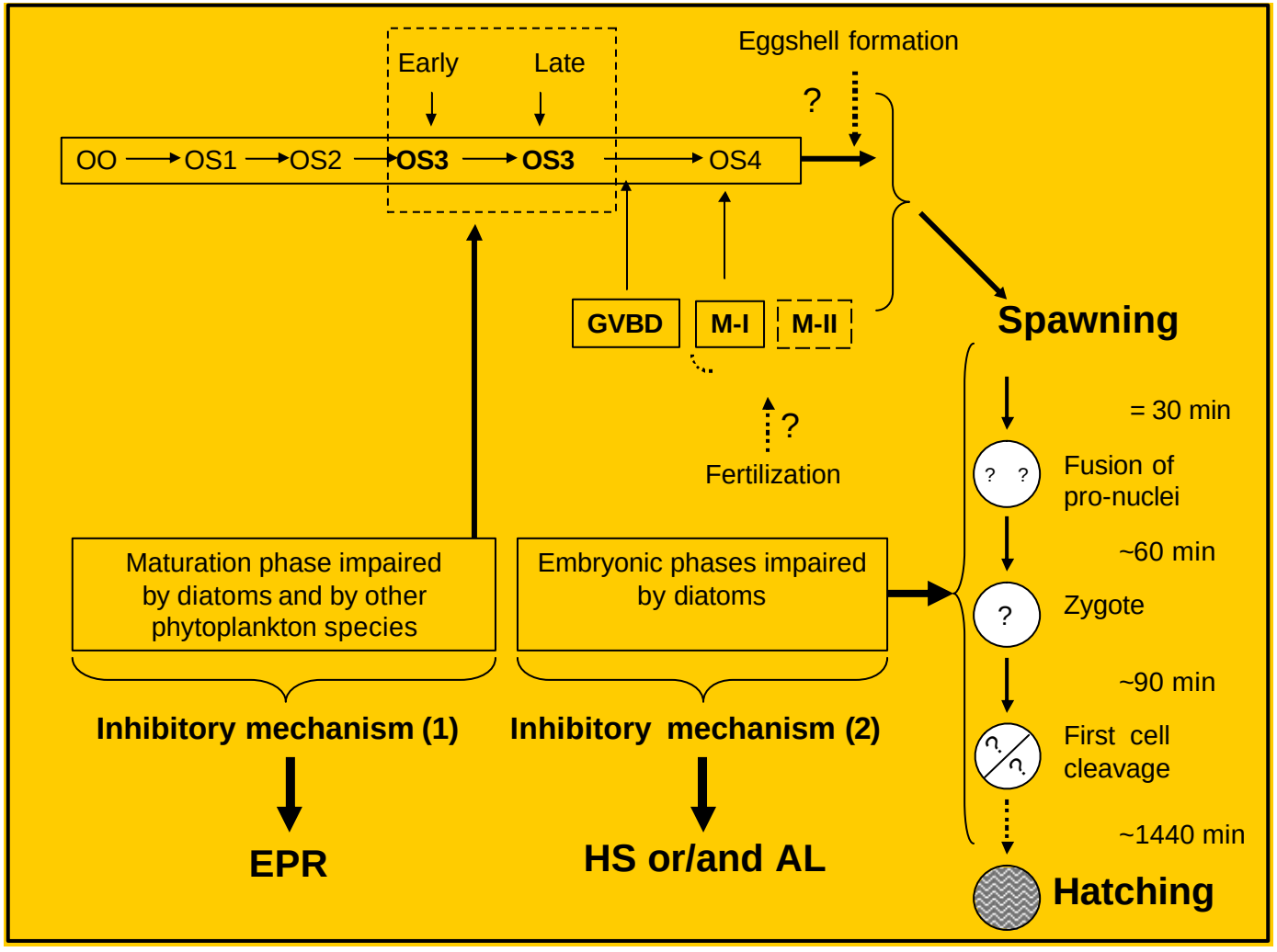
Type of diet	**PM**	**CC, GS, OR, RS, ST**	**NAV, NIT, SK, TR**	**GD, PL, TP, NDA1-7**	**STAR**
Reproductive factors					
EPR	+	-	+	-	-
HS	+	+	-	-	**?**
AL	+	+	-	-	**?**

Reference-

(1) Ban, S., Burns, C., Castel, J., Chaudron, Y., Christou, E., Escribano, R., Fonda Umani, S., Gasparini, S., Guerrero Ruiz, F., Hoffmeyer, M., Ianora, A., Kang, H-K., Laabir, M., Lacoste, A., Miralto, A., Poulet, S., Ning, X., Rodriguez, V., Runge, J., Shi, J., Starr, M., Uye, S-I., and Y. Wang. 1997. The paradox of diatom-copepod interactions. **Marine Ecology Progress series.**157:287-293.

(2) Poulet, S.A., Cueff, A., Wichard, T., Marchetti, J., Dancie, C., Pohnert, G (2006) Influence of diatoms on copepod reproduction. **III**. Consequences of abnormal oocyte maturation on three reproductive factors in *Calanus helgolandicus.* **Marine Biology**. (submitted).

Conceptual model of the inhibitory mechanisms



Reference-

S.A. Poulet, A. Cueff, T. Wichard, J. Marchetti, C. Dancie, G. Pohnert. 2006. Influence of diatoms on copepod reproduction. **III**. Consequences of abnormal oocyte maturation on three reproductive factors in *Calanus helgolandicus.* **Marine Biology**. (submitted).

III- The Phytoplankton Metabolites

Based on results accumulated in recent years, the various inhibitory symptoms observed following copepod reproduction failure are believed to be linked to different chemicals causes.

The inhibitory process (c.a defence mechanism) can be subdivided into three patterns [A, B, C , related to the deficiency of some essential compounds in diets [A], to the production of oxylipins [B], or to the activity of specific enzymes [C].

[A]- Passive inhibitory mechanism
1- Deficiency of polyunsaturated fatty acids (EPA, DHA)
2- Lack of Cholesterol in diet

[B]- Active inhibitory mechanism
3- Production of PUAs by specific diatoms

[C]- Indirect inhibitory mechanism
4- Diatom lipases, lipoxygenases and presumably hydroperoxide lyases activity in copepod gut

References-

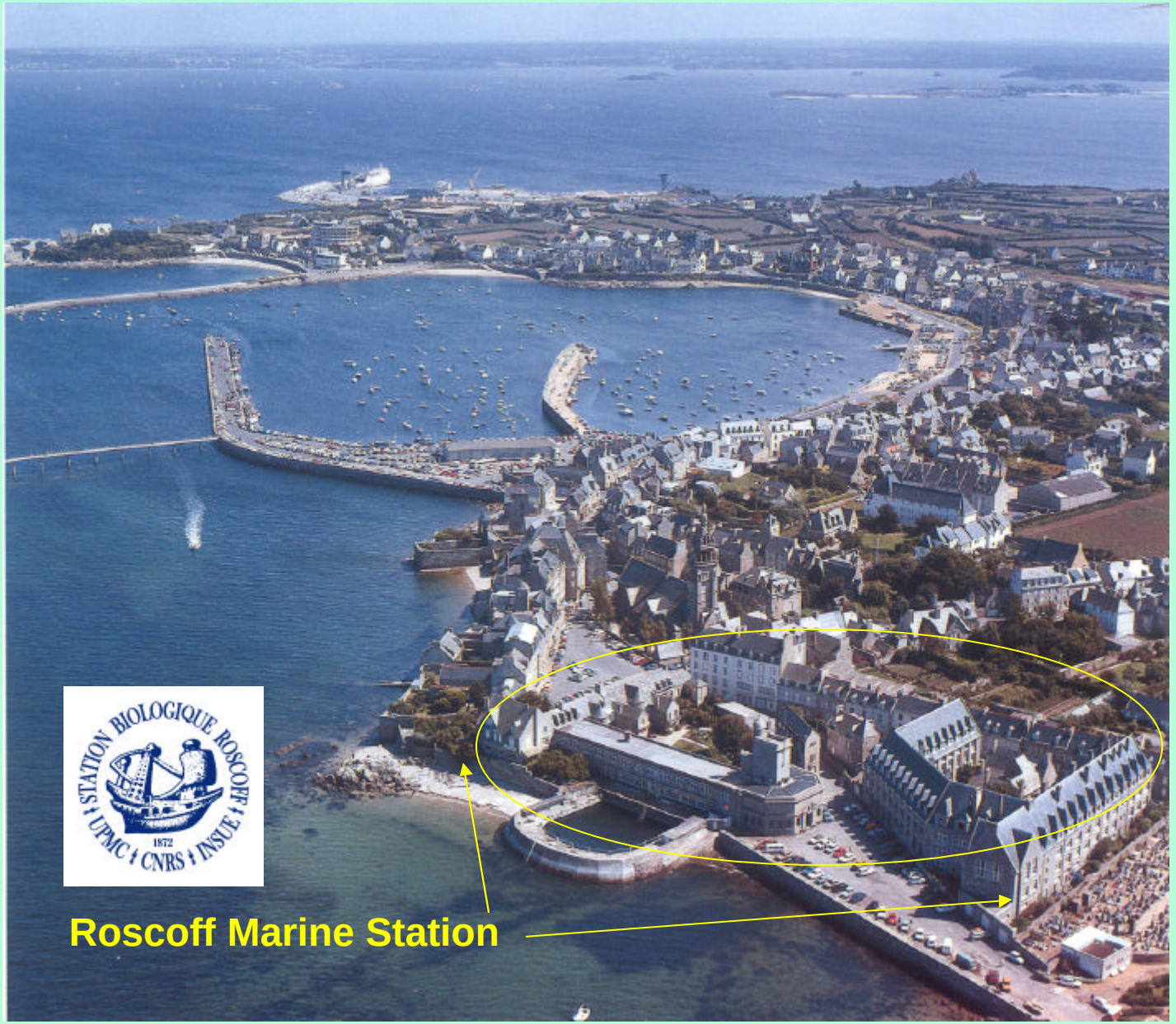
(**1**)-Jónasdóttir SH (1994) Effects of food quality on the reproductive success of *Acartia tonsa* and *Acartia hudsonica*: laboratory observations. Mar Biol 121: 67-81

(**2**)-Jonasdottir-SH; Trung-NH; Hansen-F; Gartner-S (2005) Egg production and hatching success in the calanoid copepods *Calanus helgolandicus* and *Calanus finmarchicus* in the North. J Plank Res 27: : 1239-1259

(**3**)-Hasset RP (2004) Supplementation of a diatom diet with cholesterol can enhance copepod egg-production rates. Limnol Oceanogr 49: 488-494

(**4**)-Pohnert G (2005) Diatom/Copepod interactions in plankton: The indirect chemical defense of unicellular algae. Chem Bio Chem 6: 946-959.

(**5**)-T. Wichard, A. Gerecht, M. Boersma, S. A. Poulet, G. Pohnert. Lipid and fatty acid composition of Diatoms revisited: Rapid wound activated change of food quality parameters (In prep.).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PRESENTATION N° II

BIOASSAY METHODS TO STUDY THE EFFECTS OF DIATOM METABOLITES ON THE REPRODUCTIVE BIOLOGY OF COPEPODS

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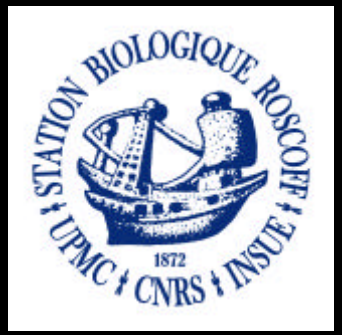


Classic methods used to measure Reproductive Rates

Tool N°1 – A boat to go offshore

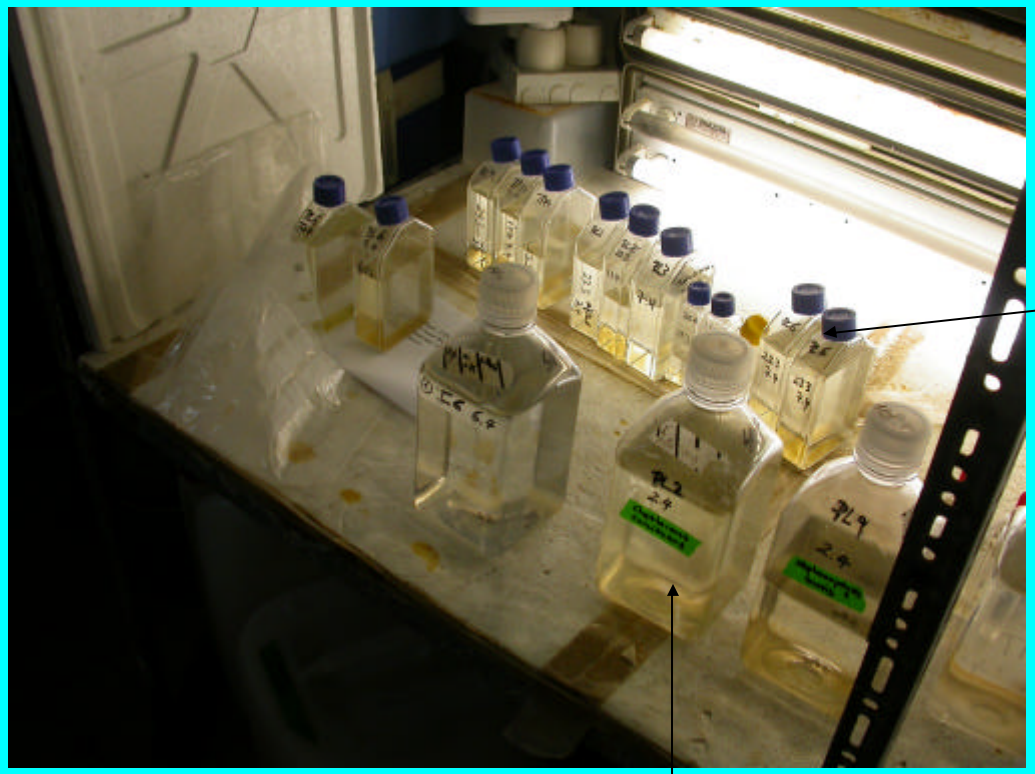
Tool N° 2- A plankton net to capture live copepods

Classic methods used to measure Reproductive Rates

Tool N° 3- The secret weapons

Classic methods used to measure Reproductive Rates

Tool N° 4- The delicious diets



Disposable vials
(50 to 250 mL)
For diatom cultures

Autoclave compatible1 L vial for phytoplankton
Diatom Cultures (food for Calanus females)

Classic methods used to measure Reproductive Rates

Tool N° 5- Hot men in a Cold Room

Adding FSW & test Diet in the incubators

Classic methods used to measure Reproductive Rates

Tool N° 6- Many vials to incubate spawing females

Classic methods used to measure Reproductive Rates

The End- Counting & sampling & incubating eggs

(Hot man and cold girl in the torture chamber)

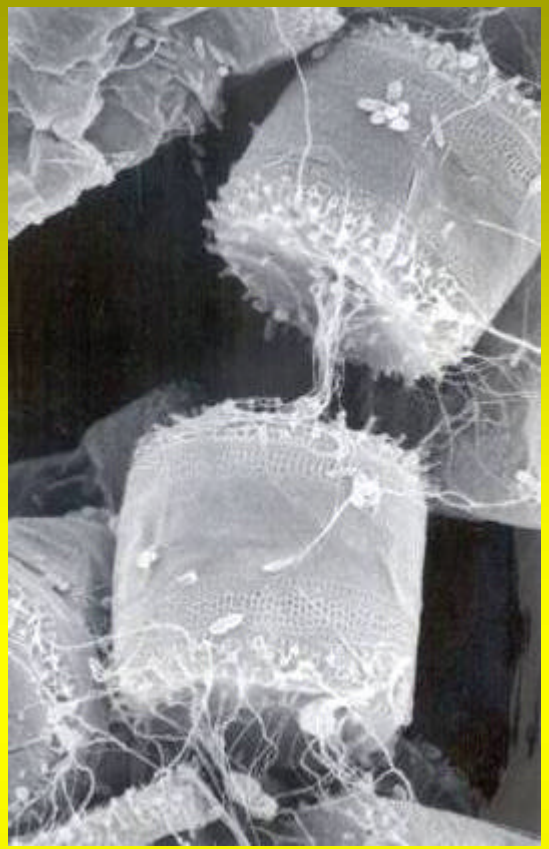
Reference-

Laabir, M., Poulet, S.A., and A. Ianora. 1995. Measuring production and viability of eggs in *Calanus helgolandicus*. **Journal of Plankton Research.** 17: 1125-1142.

Runge J.A., Roff J.C. 2000. The measurement of growth and reproductive rates. **ICES Zooplankton Methodology Manual**. Chap. 9- pp: 401-454.Academic Press.

« OTHER METHODS»

Infochemicals in diatoms



1- Detection, identification, quatification of oxylipins (PUAs)

Reference-

d'-Ippolito-G; Tucci-S; Cutignano-A; Romano-G; Cimino-G; Miralto-A; Fontana-A. 2004. The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom *Skeletonema costatum*. Bioch Biophysic Acta Mol and Cell Biol of Lip. 1686: 100-107

Wichard T., Poulet S. A., Georg Pohnert G. 2004. Determination and quantification of a, ß, ?, d- unsaturated aldehydes as pentafluorobenzyl oxime derivates in diatom cultures and phytoplankton: application in marine field studies. Journal of Chromatography. B. Analytical Technology and Biomedical Life Sciences. 814: 155-161.

2- Other potential biologically active compounds: Terpenoids

Reference-

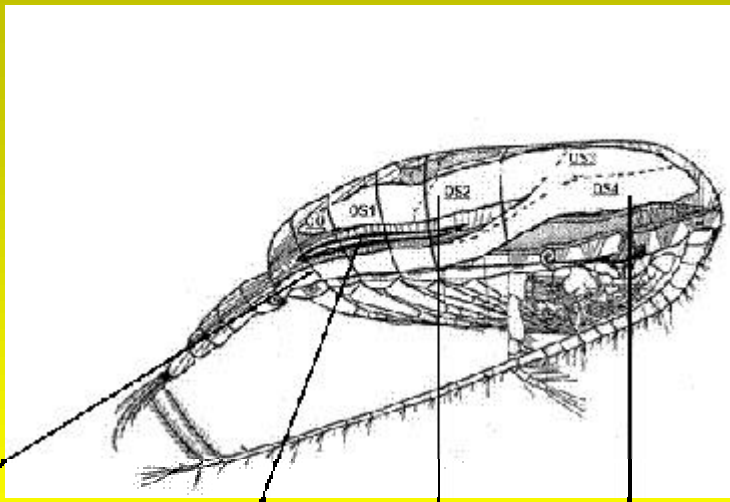
Masse-G; Belt-ST; Rowland-SJ; Rohmer-M. 2004 . Isoprenoid biosynthesis in the diatoms *Rhizosolenia setigera* (Brightwell) and *Haslea ostrearia*. Pro Nat Acad Sci. USA. 101 (13) : 4413-4418

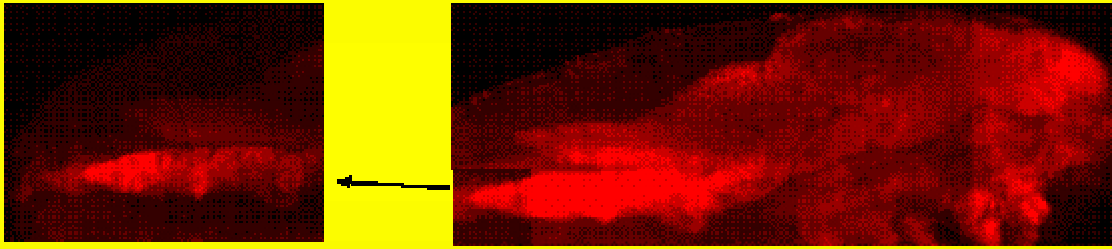
Bioassays with copepods

1- Artificial food, inert carrier with active compounds.

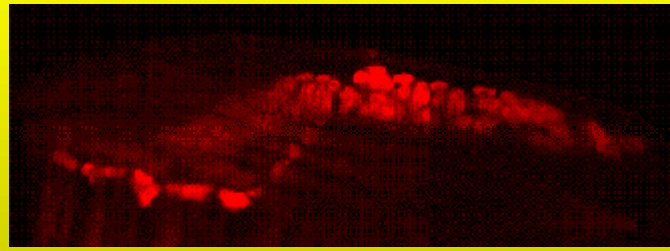
Reference-

Buttino-I; De-Rosa-G; Carotenuto-Y; Ianora-A; Fontana-A; Quaglia-F; La-Rotonda-MI; Miralto-A. 2006. Giant liposomes as delivery system for ecophysiological studies in copepods. J. Exp.Biol. 209 : 801-809.



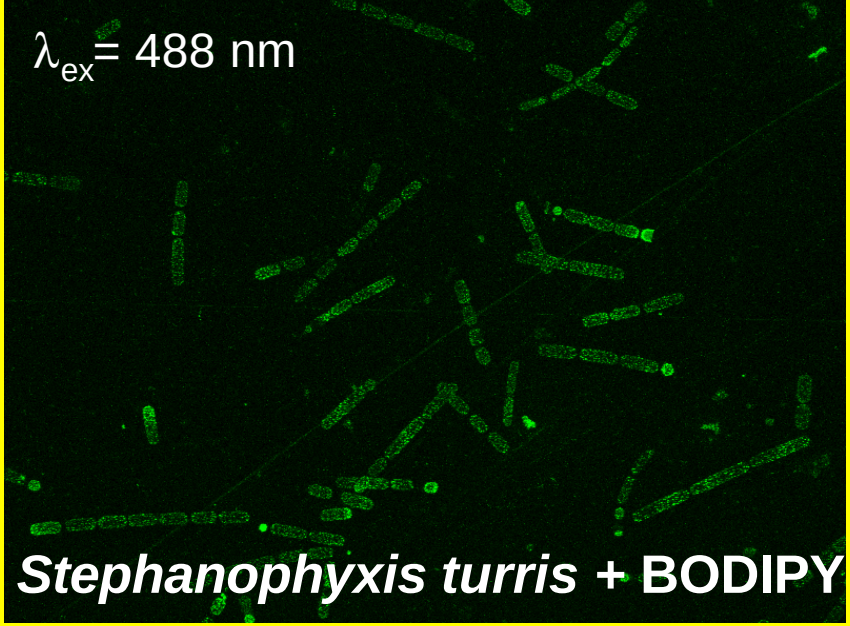


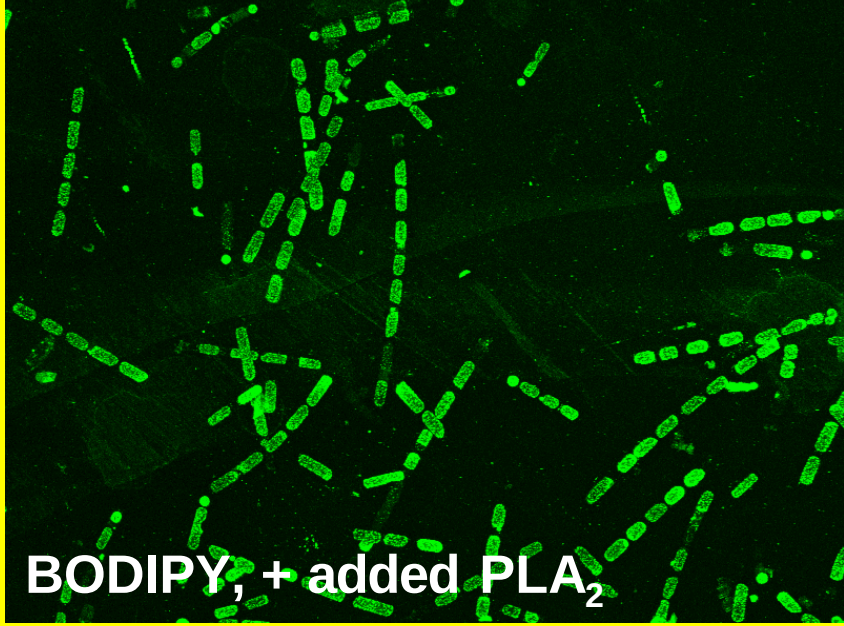
2-Microscopic observation, with ordinary UV or confocal microscope, using fluorescent markers (Tunel; An. Fi; Pi) ; (Bodipy).



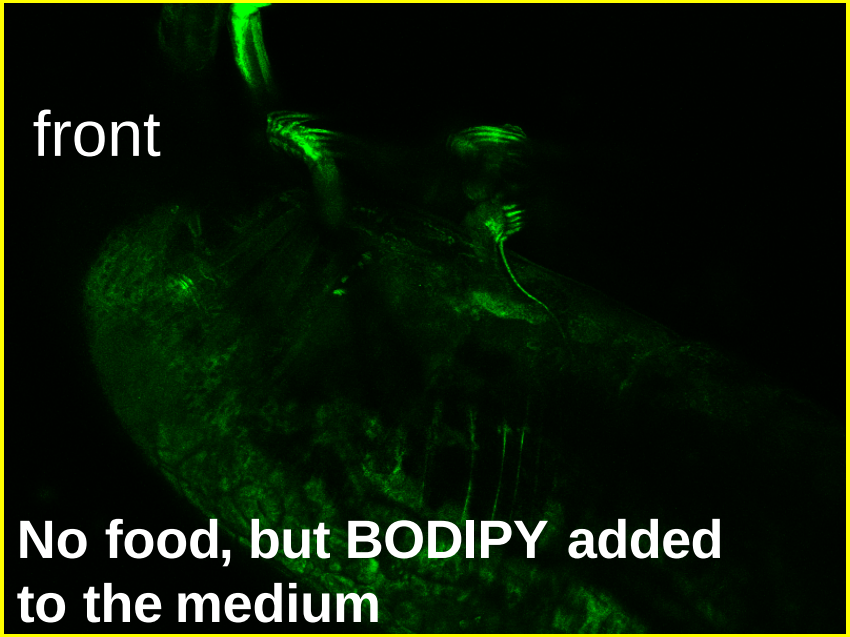
Reference-

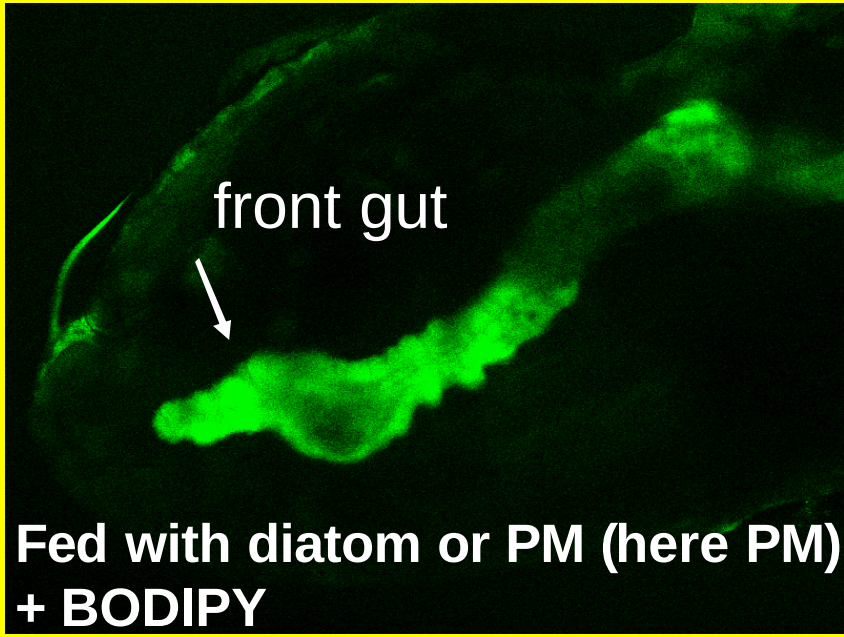
Ziessel-R; Bonardi-L; Retailleau-P; Ulrich-G. 2004. Isocyanate-, isothiocyanate-, urea-, and thiourea-substituted boron dipyrromethene dyes as fluorescent probes. J. Organ Chem. 71: 3093-3102.





Diatoms are impegnated by BODIPY





LA is active derived from diatom/PM and or copepod gut and is initiating the cascade forming aldehydes during ingest

FUTURE METHODS USED WITH COPEPODS

OBJECTIVES- Understanding the inhibitory mechanisms

Inhibition of copepod reproduction by phytoplankton diets is a complexe process.

Evidences guggest that different chemical causes are likely involved. Fatty acids (c.a EPA, DHA) and PUAs are neither directly nor exclusively involved.

It is assumed that other suspected chemical elicitor trigers one, or several specific reproductive factors(EPR, HS, AL).

A better understanding of the reproductive process in copepods will request new approaches permitting to define which inhibitory mechanism is involved.

Depending on what kind of inhibitory mechanism will be studied, different approaches can be be used.

Approach N°1-

Mechanism related to Detoxification Process

Daphnia sp.- Not affected by diatom-PUA producers **(1)**

Copepods- Partially affected by diatom-PUA producers **(2)**

} **Why ?**

Hypothesis N° 1-

Detoxification system does not exist (c.a genetic implication), or is inhibited (c.a phenotype implication) in copepods.

A key molecule involved in the Detox. Process is **Glutathione**.

Suggested Method to measure glutathion variations in copepods **(3)**

Reference-

(1) Carotenuto, Y., Wichard, T., Pohnert, G., Lampert, W., 2005. Life-history responses of *Daphnia pulicaria* to diets containing freshwater diatoms: Effects of nutritional quality versus polyunsaturated aldehydes. Limnol. Oceanog. 50: 449-454.

(2) Ianora, A., et al. 2004. Aldehyde suppression of copepod recruitment in blooms of an ubiquitous planktonic diatom. Nature. 429: 403-407.

(3) Castro-Perez-J; Plumb-R; Liang-L; Yang-E. 2005. A high-throughput liquid chromatography/tandem mass spectrometry method for screening glutathione conjugates using exact mass neutral loss acquisition. Rapid Com in Mass Spec. 19: 798-804.

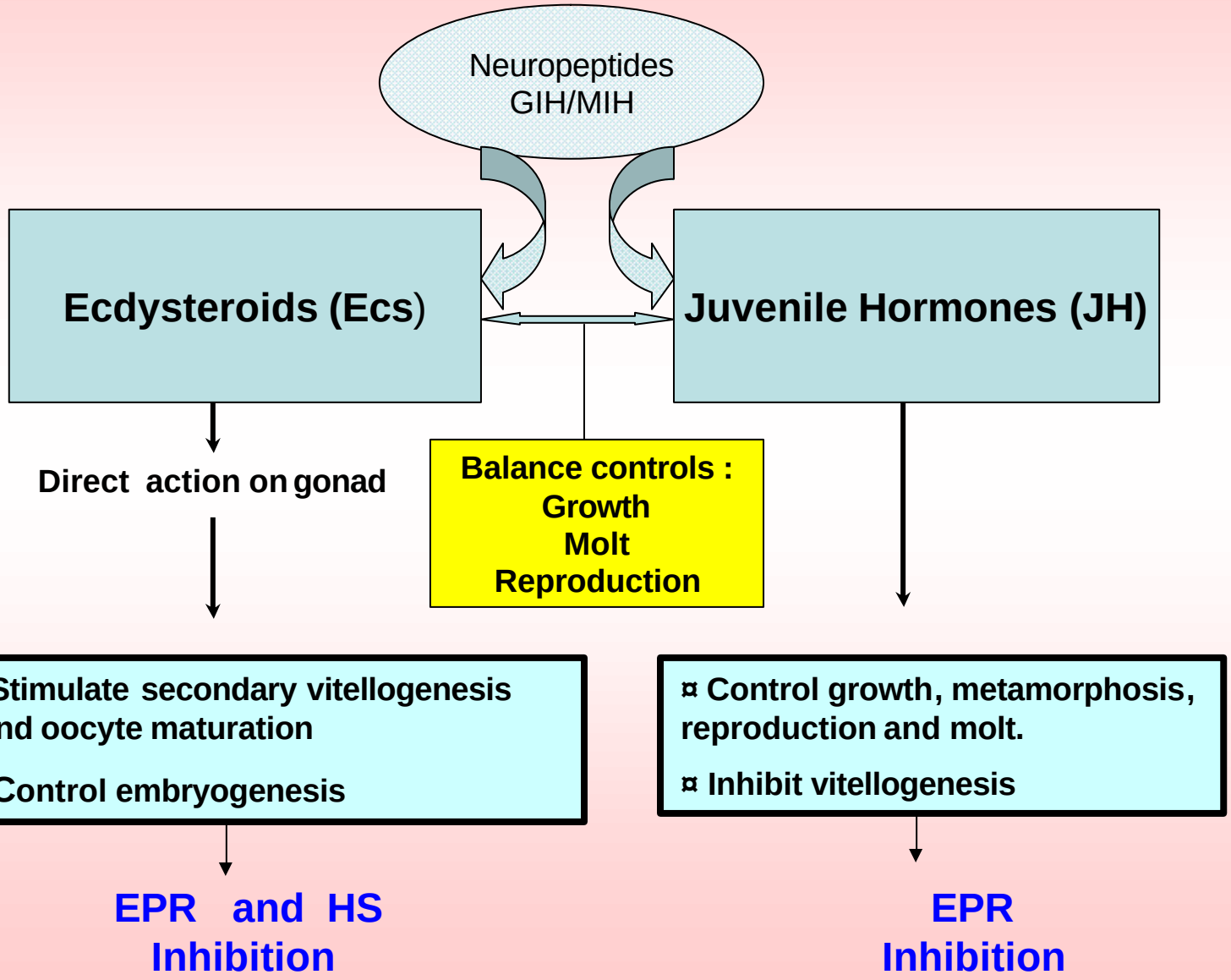
Approach N° 2-

Mechanism related to Hormone Metabolic Pathway

Hypothesis N° 1-
Inhibition of EPR (egg production rate) and/or HS (hatching success) by diets ingested by female copepds, might be due to :

A- Deficiency of hormone precursors in food, or
B- Blockage of the copepod endocrine system by specific diatom-derived toxines.

Hormones in Crustaceans & Insects Related to Reproductive Factors



Suggested Methods-

(1)- Micellar electrokinetic chromatography (MEKC)
(2)-Immuno-enzymological assays (EIA)
(3)-Radio-Immuno assay (RIA)

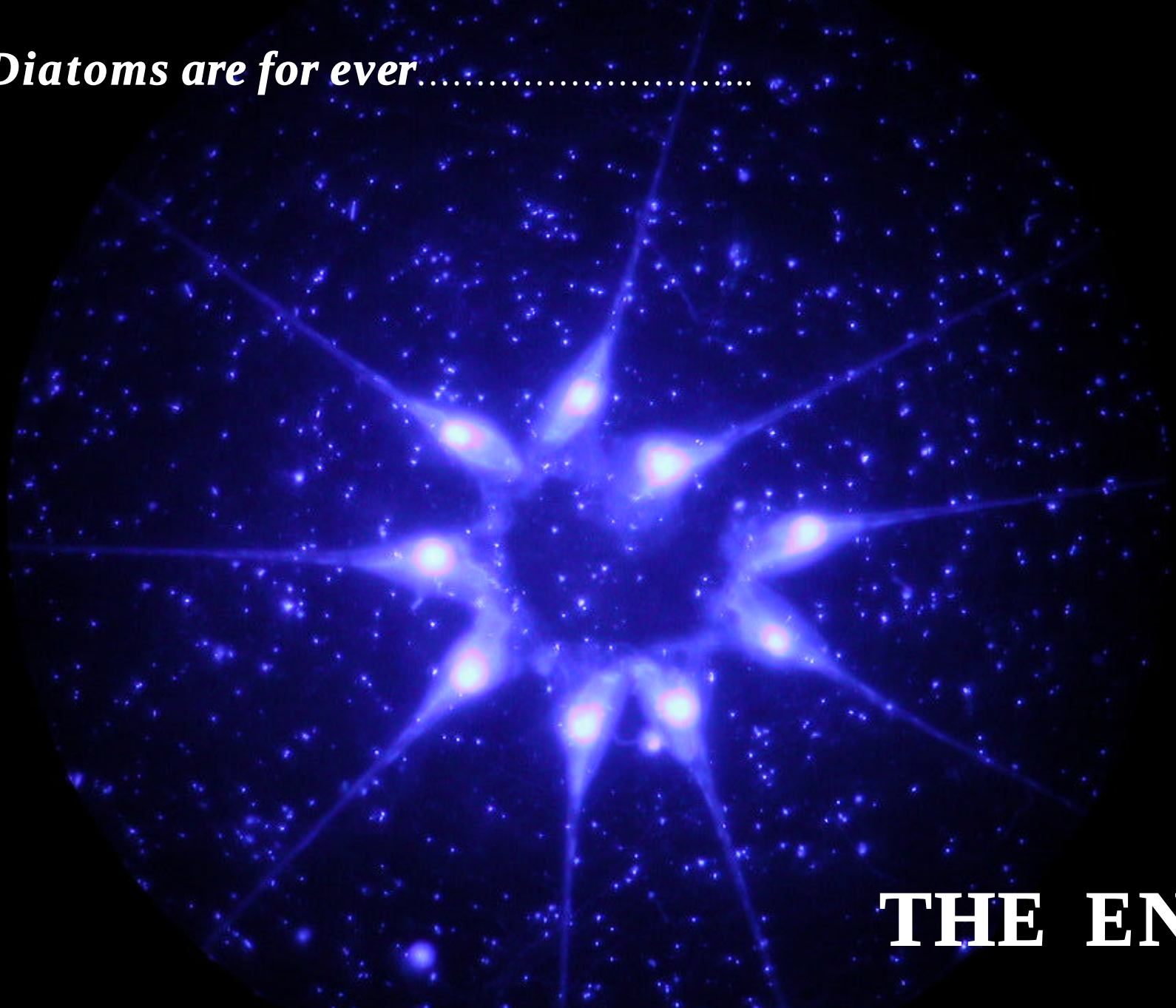
Reference-

(1)-Santagati NA, Tropea S, Ronsisvalle G. 2005. Analysis of ecdysteroids by micellar electrokinetic chromatography with on-line preconcentration. J Chromot-A. 1081: 77-86

(2)-Pascual N, Belles X, Delbecque JP, Hua YJ, Koolman J. 1995. Quantification of ecdysteroids by immunoassay: comparison of enzyme immunoassay and radioimmunoassay. Z Natur [C]. 50: 862-867.

(3)-Johnson CL. 2003. Ecdysteroids in the oceanic copepod *Calanus pacificus*: variation during molt cycle and change associated with diapause. Mar Ecol Progr Ser. 257 : 159-165.

Diatoms are for ever……..



THE END