



*Indian J. Fish.*, 58(4) : 95-99, 2011

## Growth responses of microalgae, *Chlorella salina* and *Isochrysis galbana* exposed to extracts of the macroalga, *Hypnea musciformis*

V. S. PRAMITHA AND A. P. LIPTON

Marine Biotechnology Laboratory, Vizhinjam Research Centre of Central Marine Fisheries Research Institute, Vizhinjam - 695 521, Kerala, India  
e-mail : [liptova@yahoo.com](mailto:liptova@yahoo.com)

### ABSTRACT

The influence of methanol extract and its fractions of the marine macroalga, *Hypnea musciformis* on aquaculturally important microalgae viz., *Chlorella salina* and *Isochrysis galbana* were investigated. Lower concentrations of the extract of *H. musciformis* resulted in marginal growth gain for *C. salina* and *I. galbana*. However, the fractionated extract inhibited the growth of both microalgae tested, suggesting toxicity. Results signify the importance of macroalgal extract in regulating the growth of aquaculturally important microalgae.

Keywords: *Chlorella salina*, *Hypnea musciformis*, *Isochrysis galbana*, Methanol extract

### Introduction

Marine macroalgae represent great source of majority of complex marine natural products and could be a promising source of novel bioactive compounds. These macroalgae, better known as seaweeds, synthesize a variety of secondary metabolites with complex chemical structures and different bioactivity spectra (Kannathasan *et al.*, 2008). Heat stable allelochemicals present in the extracts are reported to exhibit allelopathic effects and this could be one of the promising mitigation strategies for harmful algal blooms (Tang and Gobler, 2011). Macroalgae are reported to be rich sources of organic and inorganic nutrients like calcium, magnesium, sodium, potassium and plant growth regulators like auxin, cytokinin and gibberlins which are found to be highly essential for the metabolic activities of microalgae and some of these compounds are further used as enrichers for the growth of microalgae in aquaculture ventures (Christobel and Lipton, 2010).

Worldwide, about 30 microalgal species are widely used in mariculture as live feed, due to the presence of long chain polyunsaturated fatty acids (PUFAs) viz., eicosapentaenoic acid (EPA) and docosa hexaenoic acid (DHA) (Watanabe *et al.*, 1989; Takeuchi *et al.*, 1990). Microalgae are indispensable for early larval stages of finfish as well as shellfish species with fastidious dietary requirements that cannot be met by formulations incorporating traditional agricultural commodity products (Sorgeloos, 1996). Species of microalgae belonging to the genera *Chlorella* and *Isochrysis* have been widely utilized in marine fish hatcheries in view of transferring the essential

fatty acids from the algae to fish larvae (James *et al.*, 1987). Considering these facts, an investigation was undertaken to evaluate the effect of *Hypnea musciformis* extract on the growth performance of the microalgae, *Chlorella salina* and *Isochrysis galbana* and the results are presented.

### Materials and methods

The red macroalga, *Hypnea musciformis* (Rhodophyta) was collected from Rameswaram/Mandapam (lat. 09° 25' N; long. 79° 20' E) regions along the south-east coast of India. Live specimens were dislodged using a metal scraper and then hand-picked. The freshly collected macroalgae were washed in filtered seawater to remove the epiphytes, sand and other extraneous materials and air-dried under shade. Completely dried material was weighed and finely ground in a mechanical grinder. The crude macroalgal secondary metabolites were extracted using methanol as per the method given in Selvin and Lipton (2004). For this, 500 g of finely powdered of *H. musciformis* was refluxed with methanol in a 5 l capacity round bottom flask. The extract was filtered and concentrated to recover the excess solvents in another distillation system and subsequently reduced to a thick viscous crude extract in a rotary vacuum evaporator (Buchi) at 40 °C. The crude methanol extract (200 mg) was further fractionated using normal phase column chromatography, C-40 silica columns (Length - 600 mm, Bore - 30 mm) of 60 - 120 µ mesh size with sintered disc and screw cock following the step-gradient solvent system from low to high polarity. The fractions obtained were again evaporated and concentrated in the rotary vacuum evaporator (Fig. 1).

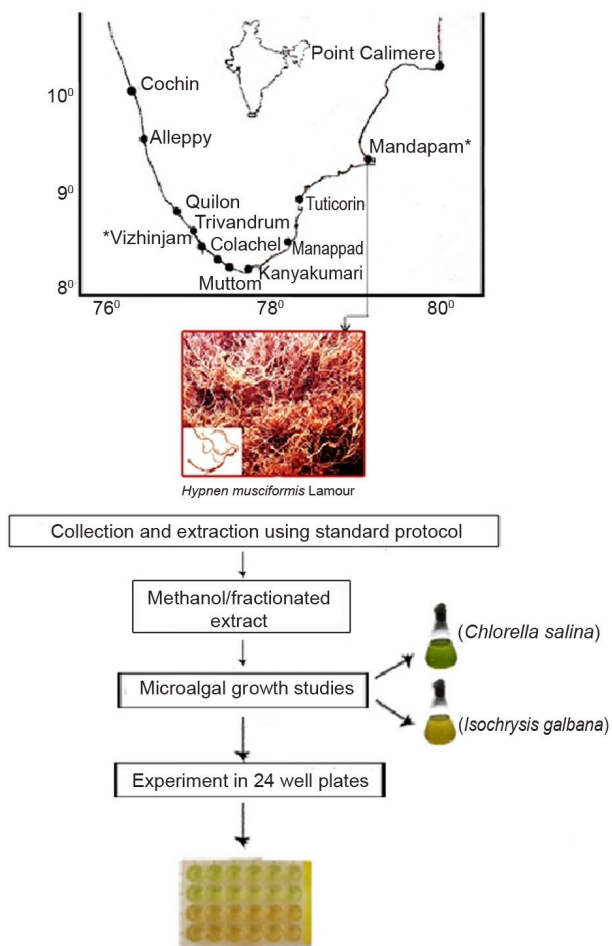


Fig. 1. Macroalgal extraction and assay procedure

Starter cultures of *Chlorella salina* and *Isochrysis galbana* were obtained from the Research Centre of the Central Marine Fisheries Research Institute, Vizhinjam and maintained in Walne’s medium at a proportion of 1 ml l<sup>-1</sup> Walne’s A, 0.5 ml l<sup>-1</sup> Walne’s B and 0.05 ml of Vitamin B<sub>1</sub> and B<sub>12</sub> (Walne, 1974). From this, 500 ml of enriched media was taken in 1 l Erlenmeyer flask and inoculated with the appropriate algal starter culture and maintained under optimum light (1000 lux) and temperature (28 ± 0.66 °C) conditions. The entire density of cells was recorded from stock culture for comparative studies.

The assay was performed in 18 mm dia 24 well plates. Selected concentrations of the extract were added to each well along with 0.2 ml of culture and the plates were incubated under illumination (1000 lux from either sides of the incubation rack) at an ambient temperature of 28 ± 0.66 °C for a period of 8 days. Walne’s medium with microalgal culture without extract served as the control. From the 1<sup>st</sup> to 8<sup>th</sup> day, the contents were stirred periodically and subsequently allowed to set for 1 to 2 h. Separate microtips were used for each sampling. The density of cells

were recorded using a haemocytometer on the 1<sup>st</sup> day, 2<sup>nd</sup> day and subsequently on every alternate days to determine the growth response *i.e.*, either proliferation or inhibition. The results were analyzed using one-way ANOVA using Microsoft Statistica Software Version 2.01.

**Results**

Addition of *Hypnea musciformis* extracts at 0.01% and 0.001% to the culture of *Chlorella salina* and *Isochrysis galbana* enhanced the growth response of cells (p<0.05). However, at 0.1% and 1.0%, inhibition of cell growth was noted (Fig. 2 and 3). The cell density of *C. salina* increased to 7.1 x 10<sup>5</sup> cells ml<sup>-1</sup> and 7.8 x 10<sup>5</sup> cells ml<sup>-1</sup> at the log phase at 0.01% and 0.001% respectively on 2<sup>nd</sup> day of culture compared to the reference culture cell density of 6.2 x 10<sup>5</sup> cells ml<sup>-1</sup> and an increase of 15 to 25% viable cell numbers was noted as compared to the control group. At the stationary phase on 4<sup>th</sup> day, in *C. salina*, 2 to 12.5% and on 6<sup>th</sup> day 9 to 13% growth enhancement was observed at

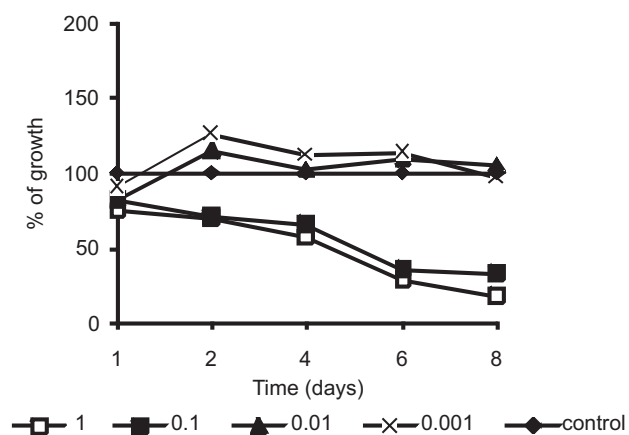


Fig. 2. Growth of *Chlorella salina* exposed to methanol extract of *Hypnea musciformis*

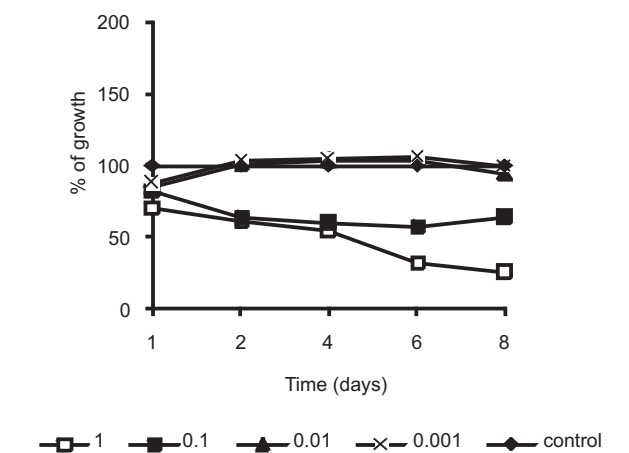


Fig. 3. Growth of *Isochrysis galbana* exposed to methanol extract of *Hypnea musciformis*

0.01 and 0.001% respectively. *I. galbana* exhibited 3 to 6% growth enhancement during the stationary phase and then declined. In *C. salina*, 1% extract level inhibited the cell growth to the extent of 24.5, 30.64, 42.22, 71.81 and 82.92% on 1<sup>st</sup>, 2<sup>nd</sup>, 4<sup>th</sup>, 6<sup>th</sup> and 8<sup>th</sup> day respectively. More or less 80% inhibition was noted at 0.1% in the stationary phase on 8<sup>th</sup> day. Upto 74.7% and 42.5% inhibition was noted in *I. galbana* at 1.0% and 0.1% level respectively.

Fractionated extract of *H. musciformis* at 0.001% and 0.01% level, inhibited the growth of *C. salina* and *I. galbana*. The 0.01% fractions of H<sub>2</sub> (Hexane - 100%), HB5a (Hexane:Benzene -80:20%), HB1a (Hexane: Benzene - 70:30%), HB2b (Hexane; Benzene 60:40%) and H<sub>4</sub> (Hexane -100%) obtained from *H. musciformis* inhibited the growth of *C. salina* to the extent of 93.44, 87.19, 80.63% on 8<sup>th</sup> day and 73 and 66.67% on 6<sup>th</sup> day (Fig. 4). The

0.001% of HB1a fraction reached declining level on 8<sup>th</sup> day post-exposure while H<sub>2</sub> showed a regaining trend from 6<sup>th</sup> day onwards towards the control level. Same trend was also shown by the fraction of HB2b at 0.001% level. H<sub>4</sub> fractions also exhibited lower level of toxicity and the growth was restored near the control group at 0.001% level (Fig. 5). For *I. galbana*, the fractions of *H. musciformis* viz., H<sub>4</sub>, HB2b, HB5a, HB1a and H<sub>2</sub> induced inhibition to the extent of 95, 90.5, 90, 77.11% on 8<sup>th</sup> day and 36.5% on 2<sup>nd</sup> day at 0.01% (Fig. 6). A drastic decline in growth of *I. galbana* with HB2b and H<sub>4</sub> at 0.01 and 0.001% was noticed from 6<sup>th</sup> day onwards. In case of H<sub>2</sub>, the growth was reduced to 7.69% on the 4<sup>th</sup> day and restored to that of the control values at 0.001% for *C. salina*. The 0.01 and 0.001% of the HB1a fraction from the 4<sup>th</sup> day onwards indicated a steady state of stationary phase up to the end of 8<sup>th</sup> day (Fig. 7).

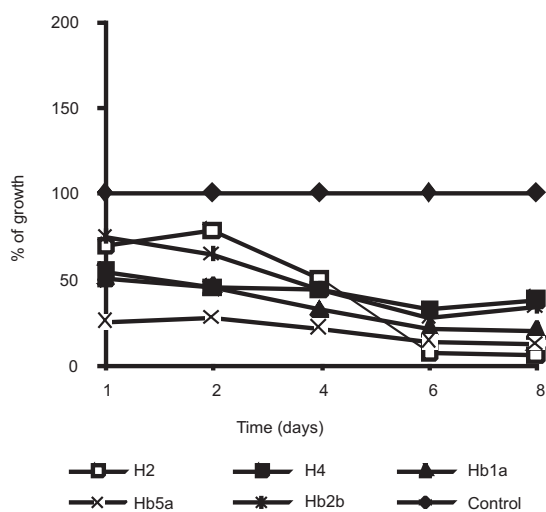


Fig. 4. Growth response of *Chlorella salina* towards fractionated extract of *Hypnea musciformis* at 0.01%.

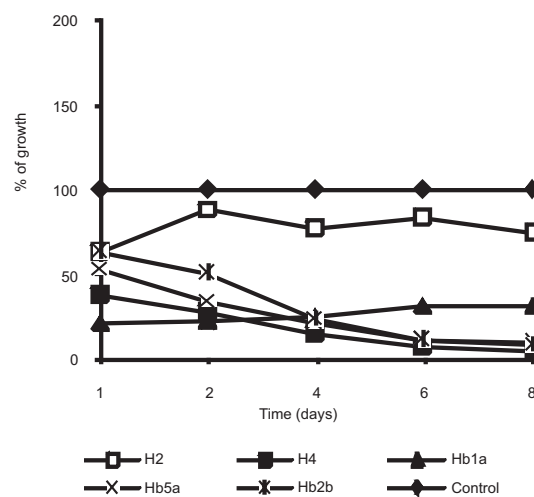


Fig. 6. Growth response of *Isochrysis galbana* towards fractionated extract of *Hypnea musciformis* at 0.01%.

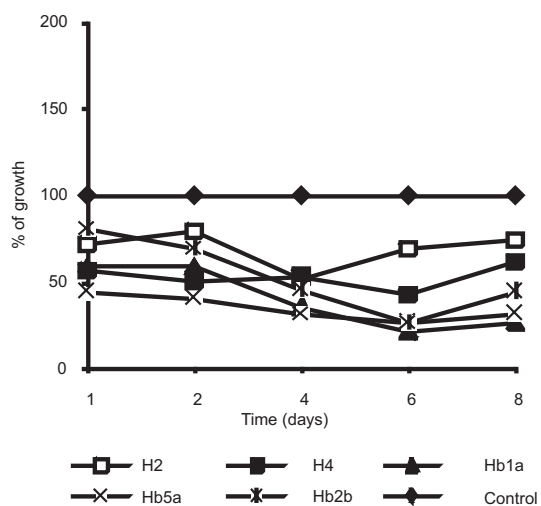


Fig. 5. Growth response of *Chlorella salina* towards fractionated extract of *Hypnea musciformis* at 0.001%.

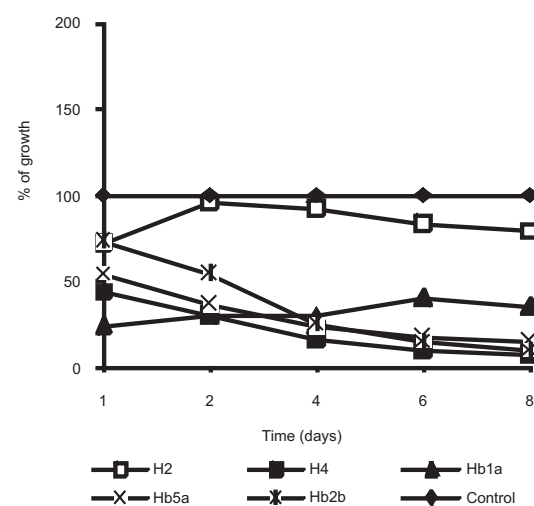


Fig. 7. Growth response of *Isochrysis galbana* towards fractionated extract of *Hypnea musciformis* at 0.001%.

## Discussion

The macroalga *H. musciformis* has been proven as a rich source of minerals and plant growth regulators (Mumford and Miura, 1998). The use of this marine alga with high protein and mineral content could be a promising way for the utilization of this marine resource that remain under-exploited along the Indian coasts (Johri and Mitra, 2001). The study was initially mooted to gain insights into the toxicity as well as difference in growth characteristics of microalgae up on supplementation of macroalgal extract in the media.

From the results, it is evident that the lower concentrations of methanol extracts of *H. musciformis* could enhance the growth of microalgae, *C. salina* and *I. galbana* at very low levels of 0.01 and 0.001% incorporation. Previous studies in our laboratory have shown that the methanol extracts of *H. musciformis* influenced the growth response of *Nannochloropsis* sp. and *C. salina* at 1 mg ml<sup>-1</sup> (Selvin, 2001). The possibilities of seaweed-based cytokinins, with the functional roles of promoting terrestrial plant growth have been reported by Sun *et al.* (1998). Cell growth stimulation of marine microalgae, *Nitzschia closterium* and *Platymonas subcordiformis* at lower concentrations of toxicants were also discussed by Huang *et al.* (2002). However, fractionated extracts of *H. musciformis* completely inhibited *C. salina* and *I. galbana* at 0.01 and 0.001%. This could be attributed to the growth regulation substances available in the extracts and the possible presence of organic compounds of pharmaceutical importance which could inhibit the growth of microalgae in the fractionated extract of *H. musciformis*.

Tang and Gobler (2011) examined the effects of the macroalga, *Ulva lactuca*, collected from estuaries of New York, USA, on the growth of seven common HAB (Harmful Algal Bloom) species *viz.*, *Aureococcus anophagefferens*, *Chattonella marina*, *Cochlodinium polykrikoides*, *Karlodinium veneficum*, *Karenia brevis*, *Prorocentrum minimum* and *Pseudo-Nitzschia multiseriata*. Their results indicated that fresh thalli of *U. lactuca* added at environmentally realistic levels (mg l<sup>-1</sup>) were capable of lysing or strongly inhibiting the growth of all seven HAB species in a dose-dependent manner within controlled laboratory experiments. The extracts of dried and powdered *U. lactuca* exhibited dramatic allelopathic effect on the HAB species.

Growth inhibitory effect of the extract of *Corallina pilulifera* towards *Cochlodinium polykrikoides* at a concentration of 50 µg ml<sup>-1</sup> compared to the reference culture level was reported by Jeong *et al.* (2000). According to Jin *et al.* (2006), the acetone and chloroform extracts of *U. pertusa* had no apparent effect on the growth of

*Alexandrium tamarense* and *Prorocentrum micans*. In the present study, all the tested fractions of macroalgal extract had inhibitory role. Kakisawa *et al.* (1988) isolated microalgal growth inhibitory substances from brown alga *Cladosiphon okamuranus* and reported that the isolated substances were active against phytoplankton without cell coverings and inactive to ones with rigid cell walls. Therefore, the difference in outermost layer structures among *Chlorella salina* and *Isochrysis galbana* cells could be attributed to their sensitivity differences to the bioactive substances of *Hypnea* sp. Kroes (1972) fractionated *Chlorococcum ellipsoideum* and found that only the lipophylic fractions had an initial inhibiting effect on *Chlamydomonas globosa* whereas the high molecular weight fraction exhibited an initial promoting effect with a subsequent inhibiting effect. Hence, the varied response characteristics of microalgae exposed to *H. musciformis* extracts could be important from the pharmaceutical as well as aquacultural point of view.

## Acknowledgements

The authors are thankful to the Director, CMFRI, Kochi for the facilities provided and the ICAR, New Delhi for the financial support

## References

- Christobel, J. G and Lipton, A. P. 2010. Influence of aqueous extract of macro alga *Hypnea musciformis* Lamour and *Codium tomentosum* (Huds) Stackhouse at different growth phases of micro algae *Chlorella marina* (Chlorophyta). In: *Proceedings of the national seminar on 'Role of environmental changes in the lower group biodiversity with special reference to algal diversity'*, February 9 - 10, 2010, WCC, Nagercoil, p. 336 - 344.
- Huang, J., Tang, X., Gong, X. and Li, Y. 2002. Preliminary study on the growth stimulation of marine microalgae stimulated by low level of toxicant. *Ying Yong Sheng Tai Xue Bao.*, 13: 1516 - 1518.
- James, C. M., Dias, P. and Salman, A. E. 1987. The use of marine yeast (*Candida* sp.) and baker's yeast (*Saccharomyces cerevisiae*) in combination with *Chlorella* sp. for mass culture of the rotifer *Brachionus plicatilis*. *Hydrobiologia*, 147: 263-268.
- Jeong, J. H., Jin, H. J., Sohn, C. H., Suh, K. H. and Hong, Y. K. 2000. Algicidal activity of the seaweed *Corallina pilulifera* against red tide microalgae. *J. Appl. Phycol.*, 12: 37-43.
- Jin, Q., Dong, S. and Wang, C. 2006. Growth inhibition to three red tide microalgae by extracts of *Ulva pertusa*. *Chinese J. Oceanol. Limnol.*, 24: 147-153.
- Kakisawa, H., Asari, F., Kusumi, T., Toma, T., Sakurai, T., Oohusa, T., Hara, Y. and Chihara, M. 1988. An allelopathic fatty acid from the brown alga *Cladosiphon okamuranus*. *Phytochemistry*, 27: 731-735.

- Kannathasan, K., Sivasankari, S., Chandrasekaran, M., Rajkumar, R. and Venkatesalu, V. 2008. Evaluation of seaweed *Hypnea musciformis* for gibberlic acid like substances and biofertilizing activity. *Seaweed Res. and Utilisn.*, 30 (1 & 2): 125-133.
- Kroes, H. W. 1972. Growth interactions between *Chlamydomonas globosa* Snow and *Chlorococcum ellipsoideum* Deasar and Bold: The role of extracellular products. *Limnol. Oceanogr.*, 17: 423-432.
- Mumford, T. F. and Miura, A. 1998. In: Lembi, C. A. and Waaland, J. R. (Eds.), *Algae and human affairs*. Cambridge University Press, Cambridge, 91 pp..
- Selvin, J. 2001. *Shrimp disease management using secondary metabolites isolated from marine organisms*, Ph. D. dissertation, Manonmaniam Sundaranar University, Tirunelveli, India, 204 pp.
- Selvin, J. and Lipton, A. P. 2004. Biopotentials of *Ulva fasciata* and *Hypnea musciformis* collected from the peninsular coast of India. *J. Mar. Sci. Tech.*, 12: 1-6.
- Sorgeloos, 1996. Intensive marine fish farming in Europe. A state of the art. In: Gajardo, G. and Coutteau, P. (Eds.), *Proceedings of the workshop on fish and mollusc larviculture*. Impresora creces, Santiago, Chile, p. 5 - 6.
- Sun, B., Fan, X. and Zhou, Y. 1998. Cytokinins: the functional bioactive composition in seaweeds. *Stud. Mar. Sing.*, 40: 139-148.
- Takeuchi, T., Toyota, M., Satoh, S. and Watanabe, T. 1990. Requirement of juvenile red seabream *Pagrus major* for eicosapentaenoic and docosahexaenoic acids. *Nippon Suisan Suisan Gakkaishi*, 56: 1263-1269.
- Walne, P. R. 1974. *Cultures of bivalve mollusks. 50 year's experience at Conway*, Fishing News (Books) Ltd., England. 173 pp.
- Watanabe, T., Arakawa, T., Takeuchi, T. and Satoh, S. 1989. Comparison between eicosapentanoic and docosahexaenoic acids in terms of essential fatty acid efficiency in juvenile striped jack *Pseudocaranx dentex*. *Nippon Suisan Gakkaishi*, 55: 1898-1995.
- Tang, Y. Z. and Gobler, C. J. 2011. The green macroalga, *Ulva lactuca*, inhibits the growth of seven common harmful algal bloom species via allelopathy. *Harmful Algae*, 10: 480-488.

Date of Receipt : 22.03.2010

Date of Acceptance : 17.12.2011