Harmful Algae
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What are the main environmental factors driving the development of the neurotoxic dinoflagellate *Vulcanodinium rugosum* in a Mediterranean ecosystem (Ingril lagoon, France)?

Abadie Eric [1, *], Chiantella Claude [1], Crottier Anais [1], Rhodes Lesley [2], Masseret Estelle [3], Berteaux Tom [1], Laabir Mohamed [3]

[1] IFREMER, Laboratoire Environnement Ressources du Languedoc-Roussillon, Centre for Marine Biodiversity, Exploitation and Conservation (MARBEC), IRD, Ifremer, CNRS, Université de Montpellier, CS30171, Sete Cedex 03, France
[2] Cawthron Institute, 98 Halifax Street East, Private Bag 2, Nelson, 7042, New Zealand
[3] Université de Montpellier, Center for Marine Biodiversity, Exploitation and Conservation (MARBEC), IRD, Ifremer, CNRS, Université de Montpellier, Place E. Bataillon, CC93, 34095, Montpellier Cedex 5, France

* Corresponding author : Eric Abadie, email address : Eric.abadie@ifremer.fr

Abstract :

Vulcanodinium rugosum, a dinoflagellate developing in Ingril Lagoon (Mediterranean, France) is responsible for shellfish intoxications due to the neurotoxin pinnatoxin G. A one year survey (March 2012–April 2013) was conducted in this oligotrophic shallow lagoon and key environmental parameters were recorded (temperature, salinity and nutrients). The spatio-temporal distribution of *V. rugosum* in water column and on macrophytes was also determined. Planktonic cells of *V. rugosum* were observed at all sampling stations, but in relatively low concentrations (maximum of 1000 cell/L). The highest abundances were observed from June to September 2012. There was a positive correlation between cell densities and both temperature and salinity. Non-motile cells were detected on macrophytes, with a maximum concentration of 6300 cells/g wet weight. Nitrite and ammonium were negatively related to *V. rugosum* abundance whereas total nitrogen, total phosphorus and phosphates showed a positive correlation. Altogether, *in situ* results suggest that *V. rugosum* is rather thermophilic and that organic nutrients should be considered when studying the nutrition requirements for this noxious expanding dinoflagellate.

Highlights

► Results indicated that temperature was the key factor influencing cell concentrations of *V. rugosum* and the development periods in this ecosystem. ► *V. rugosum* abundance and particularly that of the planktonic motile form was positively related to Total Nitrogen ,Total Phosphorus , phosphate. ► *V. rugosum* abundance (motile form) was negatively correlated with nitrite, nitrate , ammonia and Dissolved Inorganic Nitrogen. ► No significant correlation between epiphytic *V. rugosum* cell density and their substrate (macrophytes) were found.

Keywords : *Vulcanodinium rugosum*, Neurotoxins, Ingril lagoon, Environmental factors, Development dynamic

1. INTRODUCTION

Many dinoflagellate species are responsible for harmful algal blooms (HABs), which may have a negative impact on economic activities and human health (Smayda, 1997; Rhodes and Munday 2016). The "REPHY" network (French monitoring network on phytoplankton and phycotoxins), instigated in 1984, highlighted the increase in frequency of HABs and the spread of related species along the French coastline. In the French Mediterranean lagoons, many dinoflagellates are responsible for shellfish intoxications and farm closures. *Alexandrium catenella*, which produces paralytic shellfish toxins (PSTs), has bloomed regularly in Thau Lagoon since 1998 (Abadie, 1999; Laabir et al., 2011; Laanaia et al., 2013), with high densities being recorded in the water column (up to 14 x 10^6 cell/L in 2004). Thau Lagoon has experienced many toxic events with PST intoxications exceeding the sanitary threshold (800 µg Eq STX / kg fresh mollusc meat) and causing the closure of oysters farms with the subsequent economic damage as up to 10 000 tons of molluscs are produced each year in this lagoon (Laanaia et al., 2013). *Dinophysis acuminata* was also responsible for numerous events of diarrheic shellfish poisoning (DSP) toxin contamination in Leucate Lagoon, 150 km far from Thau despite this dinoflagellate having very low cell densities in water column ($2x10^2$ to $8.3x10^3$ cell/L in 2013; REPHY records). In 2007, an atypical toxicity related to mussels collected in Ingril Lagoon was detected. Mouse death symptoms due to the intraperitoneal injection of *Mytilus galloprovincialis* extract (mouse bioassay; (Yasumoto et al., 1984) differed from the classic DSP symptoms related to known lipophilic toxins (okadaïc acid, Dinophysistoxins, azaspiracids and yessotoxins) and seemed to be a neurotoxic in effect (Hess et al., 2013). This atypical toxicity was since then recurrent in this lagoon. In 2010, REPHY changed the biotoxin analysis protocol to accord with European Union regulatiory law. The mouse bioassay was replaced by a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analytical method, allowing the detailed measurement of lipophilic toxins in shellfish. This determined definitively that the known lipophilic toxins were not incriminated. Hess et al. (2013) showed that this fast acting atypical toxicity corresponded to a toxic cyclic imine group, the pinnatoxins, and that pinnatoxin G was the cause of the observed mouse symptoms in Ingril Lagoon mussels.

Pinnatoxins, first isolated from *Pinna muricata* (Chou et al., 1996; Uemura et al., 1995) were described as potent shellfish poisons. In 2010, Nézan and Chomérat (2011) isolated a dinoflagellate from Ingril Lagoon and identified this organism as a new species, *Vulcanodinium rugosum*. Smith et al. (2011) detected the same pinnatoxin G producing

dinoflagellate in Japan, and Rhodes et al. (2010, 2013), isolated New Zealand and Australian strains which produced pinnatoxins E,F and E,F,G respectively. *V. rugosum* isolated from Ingril Lagoon has also been shown to produce pinnatoxins (Abadie et al., 2015; Hess et al., 2013) and are considered responsible for the observed atypical toxicity in mice. *V. rugosum* has been observed in Mexican pacific waters (Hernandez-Becerril et al., 2013) and recently this harmful dinoflagellate was linked to human skin lesions in Cuba (Moreira et al., 2016). Many studies have clearly showed that *V. rugosum* was responsible of pinnatoxin accumulation in shellfish (Hess et al., 2013; Rhodes et al., 2011; Zeng et al., 2012).

Complex biotic and abiotic factors control the development of HAB species and the resultant blooms. In particular, water temperature and salinity are believed to influence the biology and physiology of dinoflagellates and thus their population dynamics. The growth of vegetative cells, and formation and decline of blooms, of many HAB species have been shown to be highly temperature dependant (Abadie et al., 2016; Laabir et al., 2011; Matsubara et al., 2007; Nagasoe et al., 2006; Yamaguchi & Honjo, 1989; Yamaguchi et al., 1991; Yamamoto et al., 2002). Also, temperature and salinity could modulate the dynamics of HABs by influencing the rate of dormant cells formation, their excystment and ability to inoculate the water column (Genovesi et al., 2009; Ishikawa et al., 2014; Moore et al., 2015; Ni Rathaille & Raine, 2011; Sildever et al., 2015; Triki et al., 2014).

Nutrients are among the most important factors controlling phytoplankton growth. Inorganic and organic nitrogen and phosphorus play a key role in the growth and development of HAB species and eutrophication could be responsible for the development of some HAB phytoplankton species (Anderson et al., 2002; Glibert et al., 2014; Glibert, 2005). Nutrient concentrations and ratios may influence cell growth as well as the toxicity of the blooming species. Most ecophysiological and autoecological studies have been performed on planktonic dinoflagellates, for example *Alexandrium* species (Anderson et al. 2012; Collos et al. 2004; 2007) whereas few studies have been carried out on benthic dinoflagellates (Accoroni et al., 2015a; Accoroni et al., 2011; Aissaoui et al., 2014; Almazan-Becerril et al., 2015; Cohu et al., 2013; Cohu et al., 2011; Selina et al., 2014). The influence of inorganic and organic nitrogen, and also temperature and salinity, on the growth and toxicity of *V. rugosum* has only been investigated for laboratory cultures (Abadie et al., 2015; 2016). To our knowledge, no studies have been carried out on the effects of environmental factors on the occurrence of *V. rugosum* in the natural environment. The aim of this study was to investigate *in situ* the effect of the main environmental factors (temperature, salinity, nutrients levels and macrophyte substrate)

on the occurrence and density of *V. rugosum*. The spatio-temporal distribution of this dinoflagellate in Ingril Lagoon was also determined.

2 Materials and method

2.1 Sampling area

The field survey was carried out in Ingril Lagoon (Figure 1), which is a shallow Mediterranean lagoon. This area has a surface of 685 hm² and communicates with the sea through Frontignan Harbour, through a channel named Grau. An artificial channel (the Rhône channel at Sète) divides the lagoon into two parts: North Ingril and South Ingril. The depth of Ingril Lagoon reaches a maximum of 1.7 m with mainly sandy sediments. In Ingril Lagoon, due to the shallow depth, temperature and salinity varies strongly during and between seasons. The dominant wind (N-NW) influences the sea surface temperature (SST) and this and the salinity are influenced by rain pattern and wind (SST range 0 and 28.8 °C and salinity range 7.7 and 42.7; REPHY monitoring survey 2000-2013).

2.2 Water samples

For this study, water samples were collected at 10 different stations situated in the north and the south of the lagoon (Figure 1; INST1 to INST10 - Eight stations in South Ingril and two stations in North Ingril) over a period of one year (from March 2012 to March 2013). We carried out sampling monthly except between June and September 2012 where it was performed biweekly. At each station, a water sample was taken using a submersible 12 v DC pomp (Whale GP1352) at mid-depth during 3 - 5 minutes (the water column mean depth of Ingril lagoon is 0,6m). Before pumping, the pump was calibrated to calculate the exact water volume sampled. The water was collected in a modified bottle with two walls replaced by a 20 µm pore mesh net to concentrate the micro-phytoplankton fraction. The concentrate was stored in a 50 mL polypropylene tube with 100 µL of Lugol's iodine solution. The observations of *V. rugosum* in samples were made using an Olympus IMT2 inverted light microscope. The determination of *V. rugosum* concentration was ascertained according to the Utermöhl method (Utermöhl, 1931) using a 10 mL Hydrobios chamber. During the survey at

each station, we measured the environmental parameters, temperature, salinity and oxygen with a WTW probe (LF197-S and OXI197-S).

2.2 Water samples for nutrients analysis

Nutrients were measured monthly at two stations located in the north (INST8) and the south (INST10) of Ingril Lagoon. According to results for Ingril Lagoon from the Monitoring Network RSL (Réseau de Suivi Lagunaire held by Languedoc-Roussillon authorities) these two stations are representative of the northern and southern parts of the lagoon (Derolez Valerie, 2014). The nutrient analyses targeted total nitrogen (TN), total phosphorus (TP), phosphate (PO_4), nitrate (NO_3), nitrite (NO_2) and ammonium (NH_4) . The sampled sea water in each station was filtered on boat (nylon filter porosity of 100 µm) and at thereafter in the laboratory (GF/F filter diameter 0.47 mm). Water Samples were stored at -20 °C before chemical analyses according Aminot (Aminot, 2007) method.

2.3 Macrophyte samples for the determination of dinoflagellates concentrations

Macrophytes were sampled at several stations (INST1-INST10, table 1) in Ingril Lagoon when the depth of the water column allowed their sampling. The sampled macrophyte species and the water column depth changed as a function of season. In this study, three macroalgae (*Chaetomorpha* sp., *Ulva rigida* and *Gracilaria* sp.) and one magnoliophyte (*Zostera noltii)* were sampled.

When collected, macrophytes were stored in a plastic flask (1 L) filled with filtered (0.2 µm) seawater from the sampled station in order to limit the loss of Dinophycean cells. In the laboratory, cells of benthic dinoflagellates were separated from the macrophytes according to the protocol described by Blanfuné (Blanfuné et al., 2015). The final sample was fixed with formalin (2% final concentration). The dinoflagellates were identified using an Olympus IMT2 photonic inverted microscope and their densities determined using the Uthermöl method (Utermöhl, 1931) with a 10 mL Hydrobios chamber. Results are expressed in cell/g of wet weight macroalgae.

2.4 Statistical analyses

We used for ANOVA and non-parametric tests (Spearman correlation) SIGMAPLOT 12.5 software. For multivariate analysis, the software R was used with the script "The ade4

package: implementing the duality diagram for ecologists" (Dray & Dufour, 2007). The correlations between factors were linear Pearson correlation.

3 - Results

3.1 Physico-chemical characteristics of the stations

At the stations studied in Ingril Lagoon, temperature and salinity varied widely, ranging from 4.7 °C to 29 °C and from 25.7 to 42.5, respectively. No statistical differences were found between the stations for these two environmental parameters (Kruskal-Wallis One Way Analysis of Variance on Ranks p=0.995 and p= 0.695) (Figures 2, 3). Oxygen varied from 16.6 % (saturation level, 1.15 mg/L in concentration) to 176 % (13.45 mg/L). Nutrients (NO_2, NO_3, NH_4, PO_4, TN, TP) were analyzed for only two stations in each part of the lagoon due to the hydrologically homogeneous water conditions (as confirmed by the monitoring network of French lagoons RSL : INST8 (South Ingril) and INST10 (North Ingril)). Using the Spearman test, nutrients values from INST8 and INST10 were assigned to the other stations of the same part of the lagoon (INST8 for INST1 to INST7 and INST10 for INST9). It was not possible to highlight differences between nutrients concentrations from each part of the lagoon. The TN concentrations varied from 17.21 to 42.45 µmol/L; TP ranged from 0.38 to 1.47 µmol/L and NO_2, NO_3 and NH_4 varied from 0.02 to 0.53 µmol/L, from 0.02 to 6.75 µmol/L and from 0.07 to 4.96 µmol/L, respectively. The PO_4 concentration ranged from 0.07 to 0.22 µmol/L. During this study nitrogen and phosphorus sources showed relatively low values except for NO_3 and NH_4, for which the impact of two rainfall events were noted (23/11/2012 and 26/03/2013) with subsequent measured concentrations of these two nitrogen forms were 6.75 and 4.96 µmol/L, respectively. The maximum values found in this study (Figures 4A, 4B, 5A,5B) were consistent with the values registered in RSL network.

3.2 Temporal and spatial variation of *Vulcanodinium rugosum*

3.2.1 *V. rugosum* abundance variations in water column

V. rugosum was detected at a very low concentration in March, April and May (from 3 to 25 cell/L) and in October and November (from 6 to 48 cell/L). In contrast, this species was present in relatively high densities in the water column between June and September 2012 (Figure 6) with a maximum abundance at the station INST6 in July 2012 (995 cell/L). In the water column, only motile cells were observed.

In spite of the absence of a significant statistical difference between the stations ($p = 0.241$), *V. rugosum* abundance seemed to be higher at INST5 and INST6 and, to a lesser extent, at INST8, with a maximum abundance of 490, 995 and 557 cell/L, respectively. The two parts of the lagoon (North and South) were separated by a channel. *V. rugosum* abundances in the North part of the lagoon were lower (up to 6 times) compared to those of the South part. These observed differences are statistically significant ($p<0.05$).

3.2.2 Seasonal abundance of *Vulcanodinium rugosum* attached to the macrophytes

In this study we found non-motile cells of *V. rugosum* only on the macrophytes. The abundance of *V. rugosum* cells on macrophytes varied in relation with the station and the sampling period (Figure 7). The highest abundance of cells was observed between June and August with a maximum of 6339 cell/g at INST1 in June 2012, 2654 cell/g at INST6 in July 2012 and 477 cell/g at INST9 and INST10 in August 2012.

The highest concentrations of *V. rugosum* were observed on *Chaetomorpha* sp. (INST1, INST8 with 6339 and 4377 cell/g), on *Zostera noltii* (INST6 with 2654 and 2174 cell/g) and on *Ulva rigida* (INST10 with 1026 cell/g).

We could expect link between benthic and planktonic stages. We tested relationship (linear and non linear) between the densities of planktonic and benthic forms of *V.rugosum*. We found no relationship between benthic and planktonic stages and the R-square was of 0.11 (for a linear regression ; $p=0.755$) and 0.20 (for a polynomial regression order 3).

3.3 Environmental factors in relation with *Vulcanodinium rugosum* abundance

3.3.1 Influence of the temperature and the salinity

The Spearman test confirmed the importance of the temperature controlling *V. rugosum* abundance (Table 2). The correlation coefficient r was highly significant when considering the abundance in water column instead that for cells living on the macrophyte (benthic forms) ($r = 0.658$ and 0.460 respectively and $p < 0.01$). Data showed that salinity was positively correlated to *V. rugosum* abundance in the water column and cells settled on macrophytes ($r = 0.526$ and 0.595 respectively and $p < 0.01$). In the water column, this dinoflagellate was observed since march (temperature of 14 °C) but in very low concentration (4 cell/L). *V. rugosum* densities higher than 50 cell/L were observed between 21 °C to 29 °C. The maximum concentration in water column (995 cell/L) occurred for a temperature of 23.9 °C and a salinity of 40.5. *V. rugosum* abundance on macrophytes was less correlated to temperature. This dinoflagellate was detected with densities higher than 1 cell/g WW macrophytes from March 2012 to November 2012 for temperatures between 13.8 °C and 26.6 °C and a salinity ranging between 32.4 to 41.9.

As confirmed in the Figure 8, the maximum cell concentration of *V. ru*gosum was observed during the favorable window of temperature (>20 °C). For the pelagic form the cell density increased with the water temperature until to reach the maximum of 995 cell/L.

3.3.2 Nutrient variations and *Vulcanodinium rugosum* dynamic

Data showed that NO_2, NH_4 and to a lesser extent NO_3, had a negative Spearman correlation with the *V. rugosum* abundance in water column ($r = -0.691$, -0.453 and -0.367, respectively and $p < 0.01$) (Table 2). This abundance was, however, positively correlated with TN, TP and, to a lesser extent, with PO_4 ($r = 0.469$, 0.387 and 0.302 respectively with $p < 0.01$). The Spearman test highlighted a negative correlation for the abundance of this species on macrophytes with ammonium ($r = -0.436$ and $p < 0.01$) and a positive correlation with TN and TP ($r = 0.464$ and 0.515 respectively with $p < 0.01$). The ratio N/P (with N = TN and P= TP) was negatively correlated only with the abundance of *V. rugosum* on macrophyte ($r = -0.520$ and $p < 0.01$).

3.3.3 *Vulcanodinium rugosum* dynamics related to all the environmental factors (multivariate analysis)

3.3.3.1 V. rugosum abundance in water column

The influence of water temperature and salinity on *V. rugosum* abundance (Vulcano.L; Figure 9) were tested with Principal Component Analysis. The three first axis of the Principal Component Analyses (PCA) explained 86 % of the variance. The results (Table 3) confirmed the influence of water temperature on cell concentrations in comparison to salinity (0.32 and 0.26 of correlation coefficient respectively). The score plot of *V. rugosum* abundance (Figure 10) showed the difference between periods of sampling. The water temperature clearly influenced the abundance of cells and two different periods were identified: spring and summer periods (i.e. May to September and, to a lesser extent October) and autumn and winter period (i.e. November to April). The influence of all environmental factors were tested using PCA and results are presented in Figure 9 and Table 3. Even if the correlation factors were not high, data showed the positive influence of TN and the negative correlation with NO_2 ($p<0.05$), NH_4 ($p<0.05$) and NO_3.

3.3.3.2 Abundance of Vulcanodinium rugosum attached to the macrophytes

All the environmental factors were tested with PCA to highlight any influence on *V. rugosum* densities (Vulcano.g : cell/g wet weight) of the cells living on different macrophyte species during the monitored period (Figure 11). The first three axes explained 87.66 % of the variance. Even if the water temperature and salinity remained important factors, their influence was less apparent than for *V. rugosum* abundance in water column (0.190 and 0.200 of correlation coefficients respectively, Table 4). As for *V. rugosum* developing in water column, the positive influences of TN was confirmed (0.342 of correlation coefficient and $p<0.05$). To a lesser extent, negative correlations between benthic forms of *V. rugosum* and NH_4 (0.182), NO_3 (0.157) and NO_2 (0.140) were observed but not statistically significant

(Figure 13 ; Table 4). Unlike abundances in the water column, the results did not demonstrate a significant correlation between the period of sampling and the densities of *V. rugosum* on macrophytes (Figure 12).

4 – Discussion

4.1 Is *V. rug*osum abundance related to the sampled location and season ?

This study clearly demonstrated that *V. rugosum* bloom periods followed a seasonal trend. This is in accordance with studies performed on other benthic dinoflagellate species (Accoroni et al., 2012; Accoroni et al., 2015a; Aissaoui et al., 2014; Chang et al., 2000; Cohu et al., 2011; Rhodes et al., 2000; Turki, 2005). In this study, *V. rugosum* abundance was strongly related to spring, summer and the beginning of the autumn period. This is similar to other benthic species developing in Mediterranean coastal waters, in particular *Ostreopsis* cf. *ovata* (Accoroni et al., 2015a; Blanfuné et al., 2015; Cohu et al., 2013). In a relatively small area such as Ingril Lagoon, we noticed a trend of some heterogeneity of *V. rugosum* abundance between sampling stations, although it was not significant statistically. These variations at a small scale have also been noted for *O.* cf. *ovata* by Cohu (Cohu et al., 2013; Cohu et al., 2011).

4.2 Is *V. rugosum* abundance influenced by temperature and salinity ?

V. rugosum proliferation in the water column of Ingril Lagoon was clearly related to variations in the water temperature. In this study, the temperature required for optimal development ranged from 21 °C to 28 °C. These field results are in accordance with those from controlled laboratory experiments obtained by Abadie et al. (2016), which demonstrated that the optimum temperature of *V. rugosum* growth was 25 °C. In our study, the maximum cell density (995 cell/L) was observed for a temperature of 23.9 °C and a salinity of 40.5. This value was observed when the environmental conditions were optimum for the growth rate (μ = 0.39 for temperature ~ 25 °C and salinity ~ 40 °C ; Abadie et al., 2016)

These results are also consistent with those obtained for several other benthic dinoflagellates forming blooms in the Mediterranean. For example, the favorable temperature for *O.* cf. *ovata* ranged between 22 °C to 30 °C (Aligizaki & Nikolaidis, 2006; Cohu et al., 2013; Cohu et al., 2011; Mangialajo et al., 2008). Vila et al. (2001) reported a bloom of *Ostreopsis* sp. along the

Costa Brava coast (Spain) where temperatures were higher than 18 °C. Aissaoui et al. (2014) showed that *Prorocentrum lima* developing in the Gulf of Tunis had an optimum temperature for growth ranging from 23.1 °C to 30.2 °C and Armi et al. (2010) reported a bloom of *Coolia monotis* occurring at temperatures greater than 22 °C. Results from the literature (Accoroni et al., 2015a; Cohu et al., 2013; Kibler et al., 2012; Penna et al., 2006; Xu et al., 2016) and those obtained in Ingril Lagoon, confirm that temperature is one of the most important factors explaining the proliferation of benthic dinoflagellates in Mediterranean waters, particularly when considering thermophilic species. Recently, Ben-Gharbia et al. (2016) demonstrated with laboratory experiments that three benthic dinoflagellates (*Prorocentrum lima*, *O.* cf. *ovata* and *C. monotis*) from Bizerte Lagoon, (Tunisia, South Mediterranean) had relatively high growth values at 25 °C.

In this study the Spearman correlation coefficient ($r = 0.658$ and $p < 0.01$) clearly demonstrated the significant effect of increasing temperature on the development of *V. rugosum*. As described by Rhodes et al. (2011)., the *V. rugosum* life cycle shows typical motile vegetative cells and unornamented non-motile cells. Here, the motile cells (pelagic stages) were observed in the water column whereas the non-motile cells were mainly founded on macrophytes. As for motile cells in the water column, the *V. rugosum* abundance attached to macrophytes was related to the temperature ($r = 0.460$ and $p < 0.01$). This correlation was in accordance with previous studies for other benthic species, for example, *O.* cf. *ovata*, *P. lima* and *C. monotis* developing in Mediterranean waters (Aissaoui et al., 2014; Aligizaki & Nikolaidis, 2006; Armi et al., 2010; Cohu et al., 2013; Cohu et al., 2011; Mangialajo et al., 2008), although the temperature range corresponding to the presence of *V. rugosum* on macrophytes was larger than that for the presence of this species in the water column (from 11.6 °C to 26.6 °C and from 21 °C to 29 °C, respectively). This difference between optimum ranges of temperature for pelagic and benthic forms of dinoflagellates has been reported by Vila et al. (2001) for *Ostreopsis* sp. in the in Mediterranean Spanish waters. Other studies also conducted on *Ostreopsis* species suggested an optimal temperature range between 22 °C and 30 °C, close to that found for *V. rugosum* in the present study (Aligizaki & Nikolaidis, 2006; Cohu et al., 2013; Cohu et al., 2011; Mangialajo et al., 2008). It can be hypothesized, as was demonstrated for other dinoflagellate species that *V. rugosum* benthic forms acting as temporary/pellicular cysts could better resist to bad conditions including low temperature (Onda et al., 2014; Shin et al., 2017). They give vegetative cells when the environmental conditions become favorable.

The obtained data suggested that salinity was one of the factors explaining *V. rugosum* abundance in the water column and on macrophytes (positive correlation, Spearman coefficient r = 0.526 and 0.595 respectively: $p < 0.01$). These results are in accordance with a laboratory study (Abadie et al., 2016) demonstrating that *V. rugosum* was able to grow in salinities ranging from 20 to 40 with an optimal growth rate at a salinity of 35. The laboratory study also demonstrated that *V. rugosum* could grow at a relatively low temperature (20 °C), although with a low growth rate, at salinities ranging from 35 to 40. For Mediterranean waters, Aissaoui et al. (2014) reported a bloom of *P. lima* in the Gulf of Tunis at salinities ranging from 36.1 and 40.3, with a positive correlation ($r = 0.623$ and $p< 0.05$) linking the abundance of this benthic dinoflagellate with salinity. In the same way, Armi et al. (2010) found that *C. monotis* bloomed at salinities higher than 38.6. On the contrary, Penna et al. (2006) demonstrated a negative correlation between harmful taxa density and salinity in the Adriatic Sea, but the reported taxa referred to pelagic dinoflagellates and domoic acid producing diatoms. Because of their shallow depth, the main characteristic of the French Mediterranean coastal lagoons influencing the growth of *V. rugosum* is the seasonal variation of salinity. In winter, during heavy rain events, the salinity may reach values of less than 20; whereas in summer the salinity often exceeds the salinity value of 40 (REPHY monitoring network data). For *V. rugosum*, the capacity for cells to adapt and grow at a wide range of salinities could be an advantage favoring its development in such ecosystems.

4.3 Influence of nutrients on growth of *Vulcanodinium rugosum*

Ingril Lagoon is a part of a set of eight lagoons along the French Mediterranean coast line named “Palavasiens” lagoons. These ecosystems were strongly impacted until 2005 by the wastewater treatment plant of Montpellier (MAERA). The wastewater was released after treatment (- 80 % of phosphorus and – 70 % of TN) into the Lez River, which was connected to Ingril Lagoon by the “Rhone” Channel. After 2005, the wastewater was directly released into the Mediterranean Sea. Ingril Lagoon was one of the less impacted lagoons by the MAERA wastewater plant. From 2005, the monitoring network “RSL” data (in particular during a three years survey), showed low concentrations of NO_2, NO_3, NH_4, dissolved inorganic nitrogen (DIN), TN and TP (table 5). In comparison, relatively high concentrations of nutrients were recorded for the other “Palavasiens” lagoons during the same survey (Table 5). These results suggest that Ingril Lagoon is weakly eutrophic and that the N:P ratio highlights a phosphorus limitation rather than a nitrogen limitation (RSL monitoring),

contrary to the oligotrophic Mediterranean sea (Béthoux et al., 1998). Despite the weak amounts of the measured nutrients in Ingril lagoon, *V. rugosum* developed regularly in this oligotrophic ecosystem with relatively low densities (< 6339 cell/L, Figures 6 and 7) but were able to lead to intoxications in the mussels and clams in relation to the produced pinnatoxines (Hess et al. 2013).

In this study, *V. rugosum* cell abundance in the water column and on macrophytes was related positively to TN and to TP and negatively to NO_2, NO_3, NH_4 and DIN. This would suggest that *V. rugosum* could use organic sources of nitrogen and phosphorus in natural environment. This is in accordance with Abadie et al. (2015) study showing that *V. rugosum* cultivated in the laboratory could grow in a medium with urea as the only nitrogen source, achieving a similar growth rate to when cultivated on inorganic nitrogen sources (NO_3, NH_4) and highlighting the osmotrophy of the this species. Burkholder et al., (2008) suggested that feeding on organic nutrients could give dinoflagellates a competitive advantage *in si*tu, as organic nutrients represented a significant source of nitrogen in many environments (Burkholder et al., 2008). Cohu et al. (2013) showed a negative correlation between NO_3 concentration and *O.* cf. *ovata* abundance and a positive correlation with phosphorus, while others studies (Accoroni et al., 2015a; Cohu et al., 2011) failed to find any correlation between nutrients and cell density for *O.* cf. *ovata*. Aissaoui et al. (2014) reported a negative correlation between *P. lima* density and NH_4 or N:P ratio. However a positive correlation was documented for *Prorocentrum emarginatum* abundance and NO_2. Armi et al. (2010) highlighted a positive correlation between the proliferation of *C. monotis* and NO_3. These results suggest that the nutritional response of benthic dinoflagellates, and in turn their growth, may be modulated by the trophic status of their habitats (for example, the different eutrophication levels of semi enclosed/confined areas such as lagoons or oligotrophic marine systems). In the Mediterranean lagoons, the availability of phosphorus in water appears to impact dinoflagellate development (Chikhaoui et al., 2008; Hadjadji et al., 2014; Sakka Hlaili et al., 2006). It is has been noted that the sediment of the lagoons could release phosphorus in summer due to the frequent anoxic conditions (Gomez et al., 1999; Markou et al., 2007; Zaaboub et al., 2014), which may promote dinoflagellate blooms.

4.4 *Presence of Vulcanodinium rugosum on macrophytes*

In this study, significant *V. rugosum* densities were observed on three different macrophyte species (*Chaetomorpha*, *Z. noltii* and *U. rigida with* 6339 cell/g WW, 2654 cell/g WW and 1026 cell/g WW, respectively). The lesser abundance of *V. rugosum* on *Gracilaria* (maximum of 672 cell/g) could be linked to the spatial distribution of this macroalgae or a potential allelopathic activity exerted on *V. rugosum*. It have been shown that for some benthic species, in particular *Ostreopsis*, the nature of the substrate (macrophyte species) may influence the cell abundance of the settled dinoflagellates (Aligizaki & Nikolaidis, 2006; Cohu et al., 2013; Mangialajo et al., 2008; Vila et al., 2001). In our study the difference between the abundances of non-motile cells of *V. rugosum* on the macrophytes was not statistically significant.

Even if we could expect a correlation between planktonic and benthic stages, we could not demonstrated a statistically significant relationship. This could be explained by a time gap between the presence in the same time in the planktonic and benthic forms, in the water column and on the macrophytes, respectively. The natural heterogeneity in the distribution of *V. rugosum* cells could also explain this lack of correlation.

The influence of the nature and the available surface of the macrophyte could impact the colonization of each macrophyte by this dinoflagellate. The allelopathic interactions between the macrophytes and the microalgae must also be taken into account (Laabir et al., 2013; Accoroni et al., 2016, Ben Ghrabia et al. 2017).

4 - Conclusion

This study demonstrated that *V. rugosum* was spatially distributed in Ingril Lagoon with higher abundances occurring in the south of the lagoon. Except for two stations (INST3 and INST10), the *V. rugosum* abundance in the water column was related to the period of sampling, with the highest densities observed in summer season from June to September. Results indicated that temperature was the key factor influencing cell concentrations of *V. rugosum* and thus determining the bloom event periods in this Mediterranean ecosystem. V. rugosum abundance, especially the motile form was also positively related to TN, TP and phosphate, and negatively correlated with NO_3, NO_2, NH_4 and DIN. This supports the hypothesis that growth of this dinoflagellate could be influenced by dissolved organic nitrogen.. *Alexandrium catenella* blooming in a nearby Thau Lagoon was demonstrated to be osmotrophic using urea (Collos et

al. 2004; 2007). The abundance of the non-motile cells of *V.rugosum* was not related to the macrophyte species (p=0.355). A more extensive field survey and laboratory macrophytes/*V. rugosum* co-incubation experiments have to be performed to investigate this relationship including a potential allelopathic effect of the macrophytes on this dinoflagellate. (Accoroni et al., 2015b; Chen et al., 2015; Laabir et al., 2013). The co-occurrence of *V. rugosum* with other phytoplankton species have to be investigated. The present field study highlighted the key role of temperature and salinity in determining the proliferations of *V. rugosum* in this Mediterranean ecosystem. Our data suggested that *V. rugosum* is a thermophilic and mixotrophic organism. This is in accordance with results from ecophysiological laboratory experiments performed on the same species isolated from Ingril (Abadie et al., 2015; Abadie et al., 2016). Further studies have to examine whether resistant cysts of *V. rugosum* occurred in the sediment and whether they play a role of in the bloom dynamic of this dinoflagellate. The occurrence of this neurotoxic dinoflagellate will also be investigated in other Mediterranean lagoons, such as Thau Lagoon, where important economic activities such as tourism and oyster farming (with a production of up to 10 000 tones/year) could be negatively impacted by the proliferation of *V. rugosum*.

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Tables

Table 1 : Macrophytes found at the sampling stations during the field survey including the macroalgae *Chaetomorpha sp.* (Chae), *Ulva rigida* (Ulri), *Gracilaria sp.* (Grac) and the magnoliophyte *Zostera noltii* (Zono).

	INST1	INST2	INST3	INST4	INST5	INST6	INST7	INST8	INST9	INST10
15/03/2012				Chae				Chae	Ulri	Ulri
26/04/2012				Chae				Chae		Ulri
24/05/2012				Chae				Chae	Ulri	Ulri
14/06/2012	Chae					Zono				Ulri
12/07/2012	Grac				Grac	Zono		Chae		Ulri
26/07/2012	Grac					Zono				Ulri
16/08/2012	Grac	Grac					Chae	Chae	Ulri	Ulri
19/09/2012				Chae		Zono				
05/10/2012	Grac					Zono				Ulri
23/11/2012	Grac									Ulri
01/02/2013		Chae				Zono				
28/02/2013						Zono			Ulri	
26/03/2013			Grac		Grac					Ulri

Table 2 : Spearman correlation coefficient between *Vulcanodinium rugosum* abundance (cell/L) in the water column or on the macrophytes (cells per gram Wet Weight : cell/g WW) and environmental factors in Ingril Lagoon, France. Values are in bold and underlined when significant with $p < 0.01$ and only in bold with $p < 0.05$.

	Temp	Sal	NO_2	NO_3	NH_4	DIN	PO_4	TN	TP	N:P ratio
V. rug cell/L	**0.658**	**0.526**	**-0.691**	**-0.367**	**-0.453**	**-0.441**	**0.302**	**0.469**	**0.387**	-0.125
V. rug cell/g	**0.460**	**0.595**	**-0.369**	0.137	**-0.436**	**-0.372**	**0.378**	**0.464**	**0.515**	**-0.520**

Temp: temperature; Sal: salinity; NO_2: nitrite; NO_3: nitrate; NH_4: ammonium; DIN: dissolved inorganic nitrogen; PO_4: phosphate, TN: total nitrogen; TP: total phosphorus; N/P ratio with N = TN and P= TP.

Table 3: Pearson correlations matrix between the measured environmental factors and *Vulcanodinium rugosum* abundances in the water column (cells/L).

	Vulcano.L	Temp	Sal	NO_2	NO_3	PO_4	NH_4	TN	TP
Vulcano.L	1.00000	**0.32448**	**0.25605**	**-0.24353**	-0.17608	0.00201	**-0.22022**	0.18887	0.08322
Temp		1.00000	**0.67654**	**-0.63068**	**-0.54212**	**0.52675**	**-0.46479**	**0.62804**	**0.62086**
Sal			1.00000	**-0.93355**	**-0.86629**	**0.36213**	**-0.81880**	**0.25844**	**0.44592**
NO2				1.00000	**0.87556**	**-0.33740**	**0.91577**	-0.16879	**-0.3526**
NO3					1.00000	-0.20387	**0.73863**	**-0.17734**	**-0.3956**
PO4						1.00000	**-0.22117**	**0.48941**	**0.66572**
NH4							1.00000	-0.03252	**-0.2201**
TN								1.00000	**0.86517**
TP									1.00000

Temp: temperature; Sal: salinity; NO_2: nitrite; NO_3: nitrate; PO_4: phosphate, NH_4:: ammonium; TN: total nitrogen; TP: total phosphorus. Values are in bold and underlined when significant with $p < 0.01$ and only in bold with $p < 0.05$.

Table 4: Pearson correlations matrix between environmental factors and *Vulcanodinium rugosum* abundances (cells per gram Wet Weight) on the macrophytes.

	Vulcano.g	Temp	Sal	NO_2	NO_3	PO_4	NH_4	TN	TP
Vulcano.g	1.00000	0.19036	0.20064	-0.15718	-0.14014	0.01718	-0.18209	**0.34158**	0.29765
Temp		1.00000	**0.69767**	**-0.59647**	**-0.54453**	**0.45773**	**-0.51341**	**0.54584**	**0.51892**
Sal			1.00000	**-0.89859**	**-0.87035**	0.31665	**-0.79957**	0.20329	0.31822
NO2				1.00000	**0.89995**	-0.21220	**0.93070**	-0.03154	-0.14729
NO3					1.00000	0.14489	**0.78486**	-0.05623	-0.20169
PO4						1.00000	-0.13889	**0.57887**	**0.75362**
NH4							1.00000	0.03494	-0.09682
TN								1.00000	**0.89437**
TP									1.00000

Temp: temperature; Sal: salinity; NO_2: nitrite; NO_3: nitrate; PO_4: phosphate, NH_4 :ammonium; TN: total nitrogen; TP: total phosphorus. Values are in bold and underlined when significant with $p < 0.01$ and only in bold with $p < 0.05$.

Table 5: Maximum measured concentrations of the nutrients (µmol/L) in the water column in the Ingril lagoon in comparison to those registered in other neighboring Palavasien lagoons (French Mediterranean coast).

	NO_2	NO_3	NH_4	DIN	TN	TP
Ingril Lagoon	0.15	2.10	3.67	4.05	51.57	2.22
Other "Palavasiens" Lagoon	From 0 to 3.37	From 0 to 71.66	From 0 to 21.62	From 0 to 74.96	From 0 to 315.65	From 0 to 25.79

NO_2: nitrite; NO_3: nitrate; PO_4: phosphate, NH_4 : ammonium; DIN: dissolved inorganic nitrogen; TN: total nitrogen; TP: total phosphorus

Figures

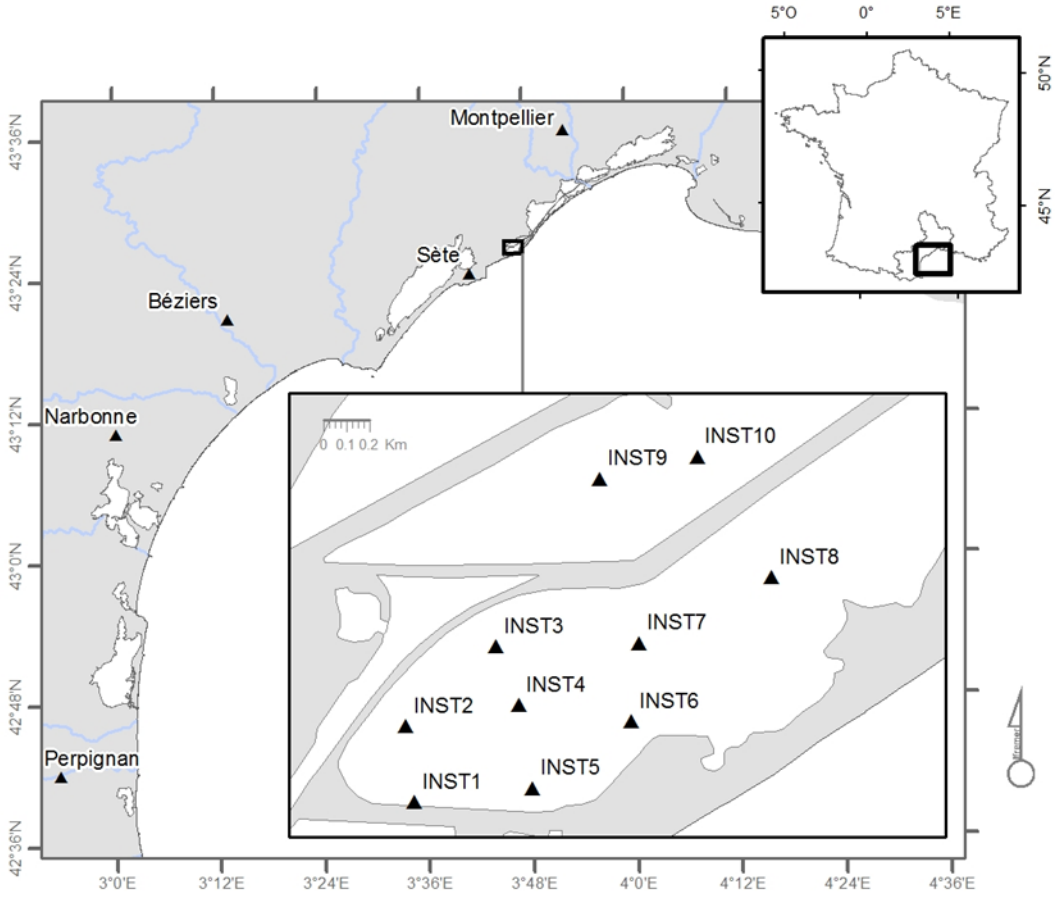


Figure 1: Sampling sites referred to in this study at Ingril Lagoon (French Mediterranean coast).

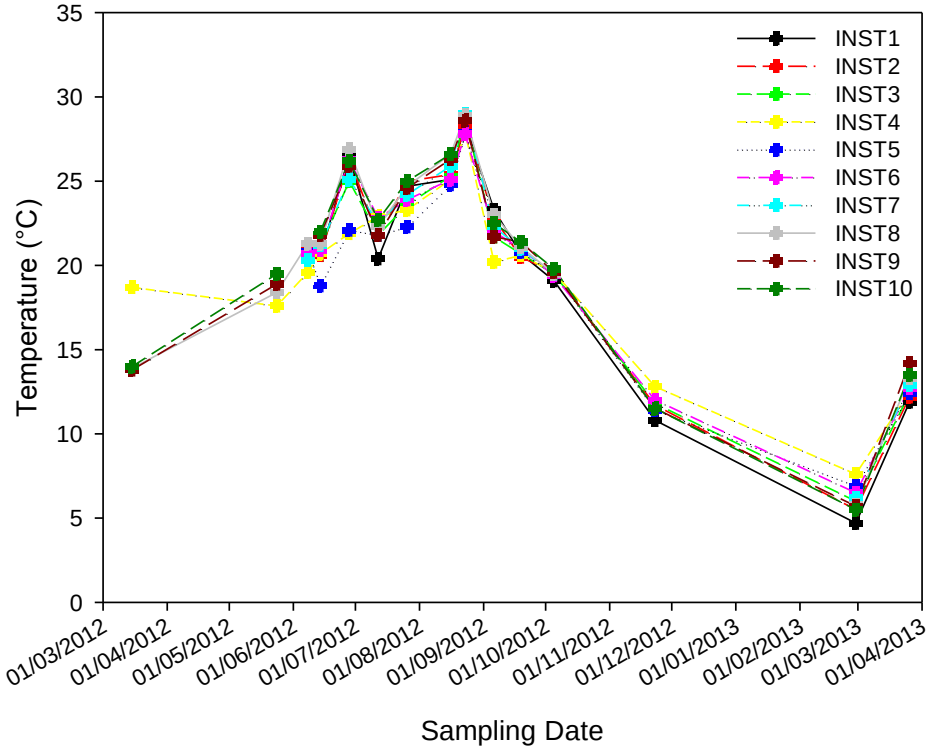


Figure 2 : Annual temperature variation in Ingril Lagoon, Mediterranean coast, France

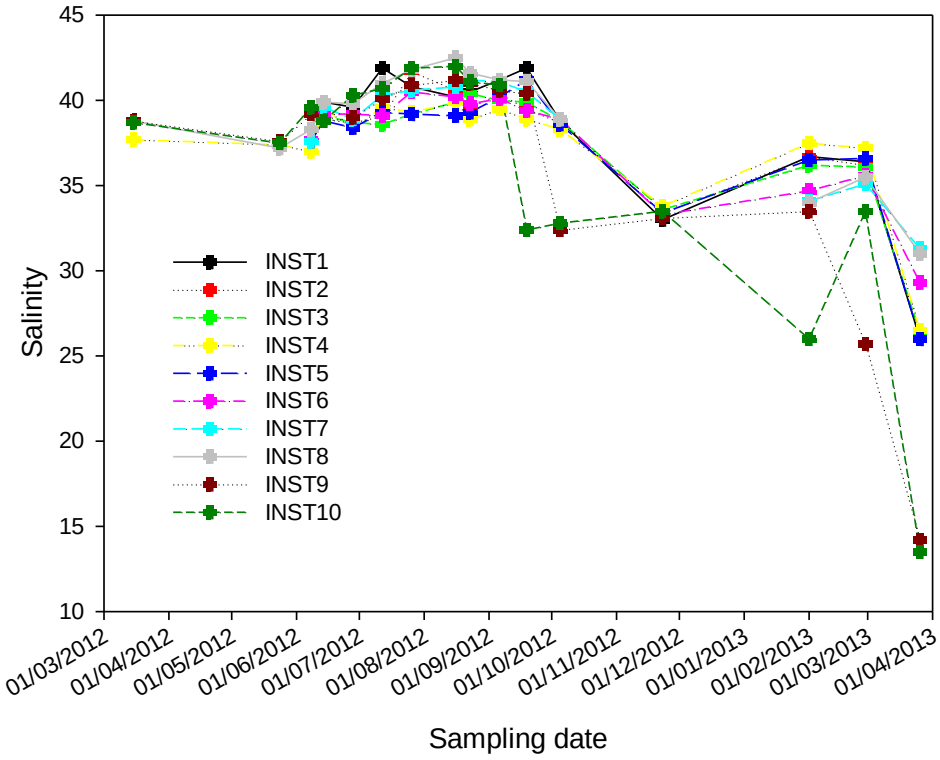


Figure 3: Annual salinity variation in Ingril Lagoon, Mediterranean coast, France

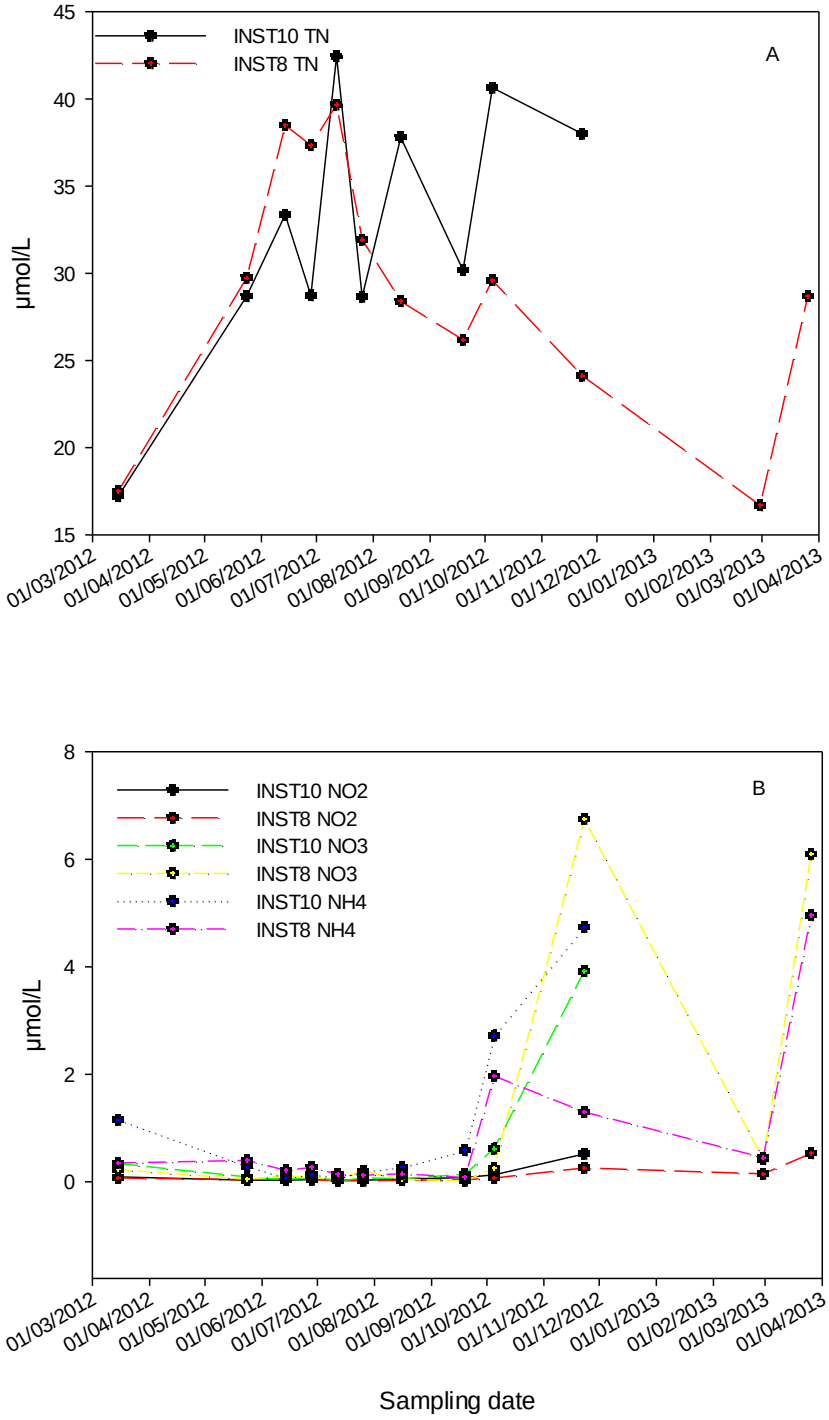


Figure 4 : Temporal variation of total nitrogen (TN – Fig.4A) and nitrate (NO_2), nitrite (NO_3) and ammonium (NH_4) (Fig.4B) at Ingril Lagoon, Mediterranean coast, France

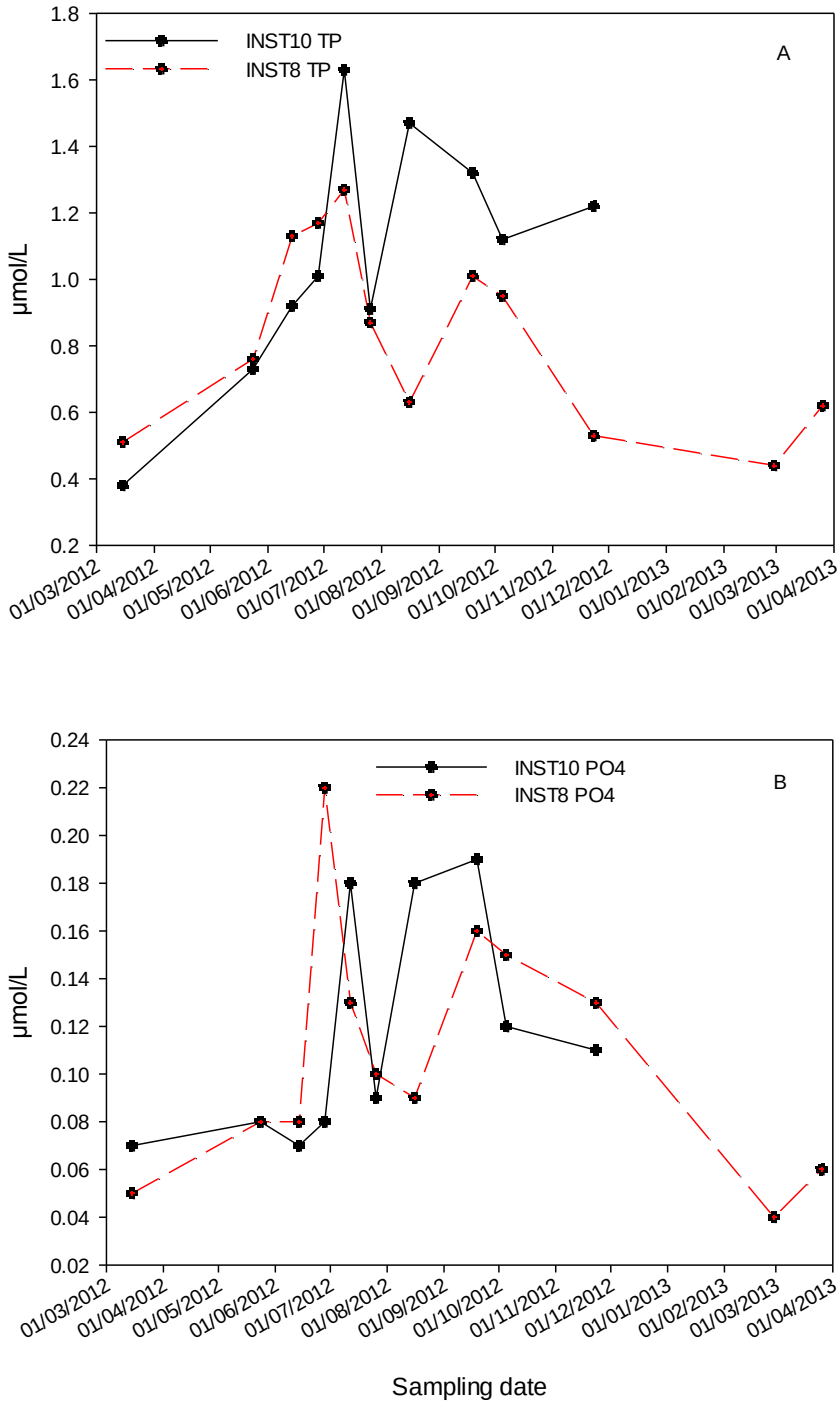


Figure 5: Temporal variation of total phosphorus TP (Fig.5A) and phosphorus PO_4 (Fig.5B) at Ingril Lagoon, Mediterranean coast, France

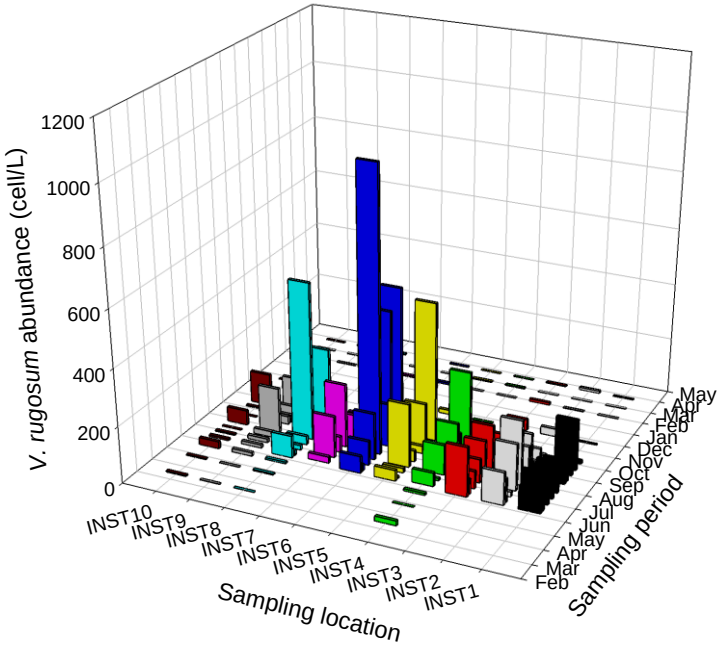


Figure 6 : Spatio-temporal abundance of *Vulcanodinium rugosum* in the water column during one year survey (March 2012 to April 2013) at Ingril Lagoon, French Mediterranean.

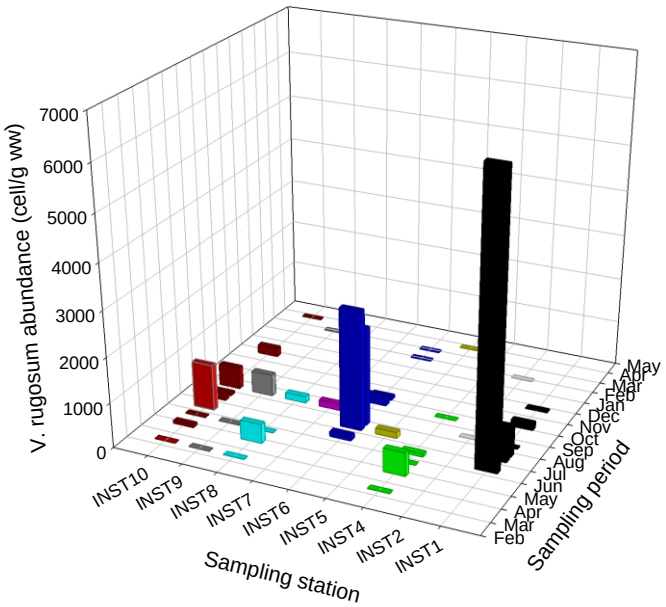


Figure 7: Spatio-temporal variations of the abundance of *Vulcanodinium rugosum* on the macrophytes during one year survey (March 2012 to April 2013) at Ingril Lagoon, French Mediterranean.

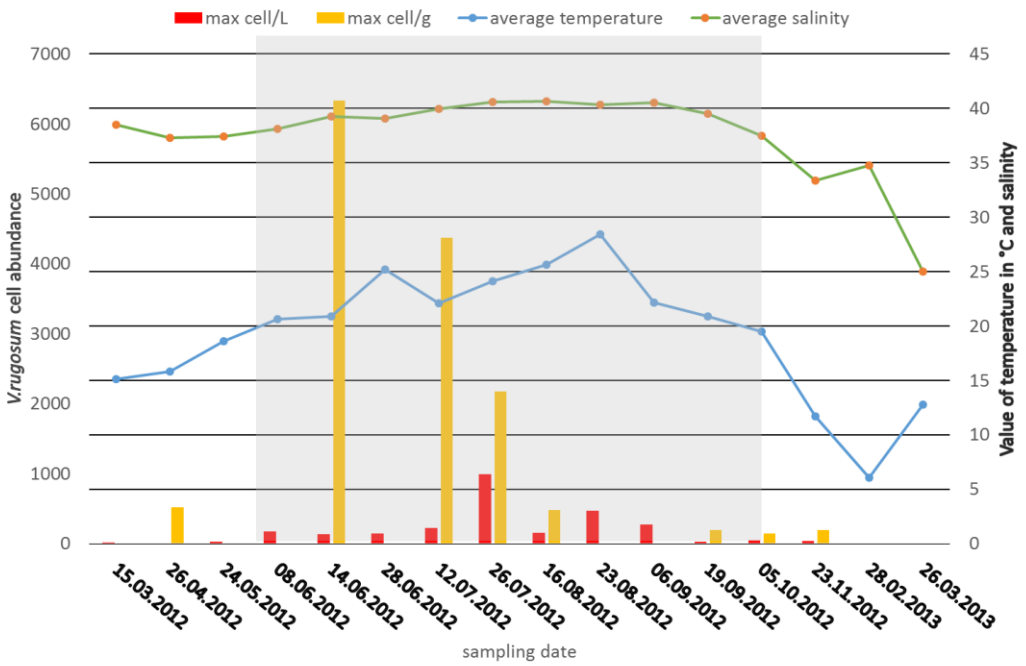


Figure 8: Maximum abundance of *Vulcanodinium rugosum* in the water column (cell/L) and on the macrophytes (cell/ g wet weight). The water temperature and salinity plotted are the average of the monthly values of the ten stations.

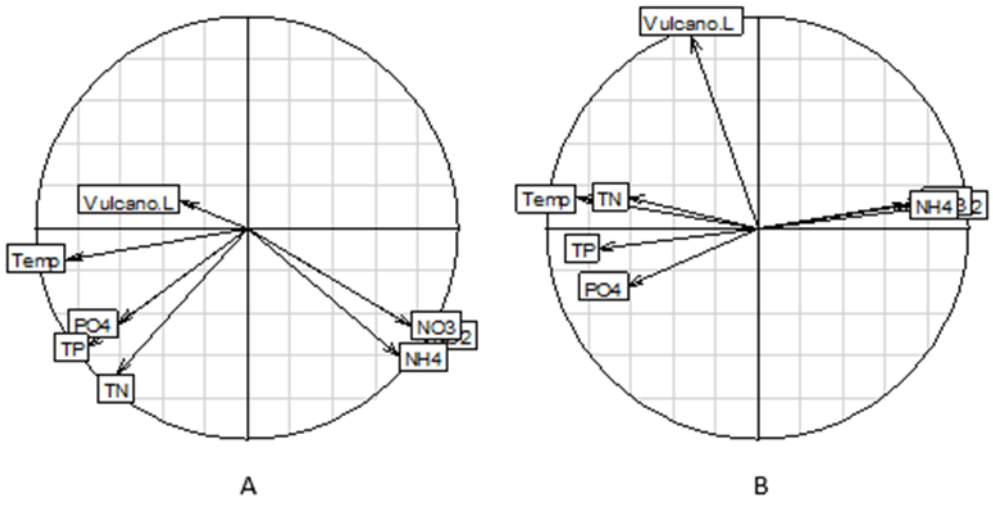
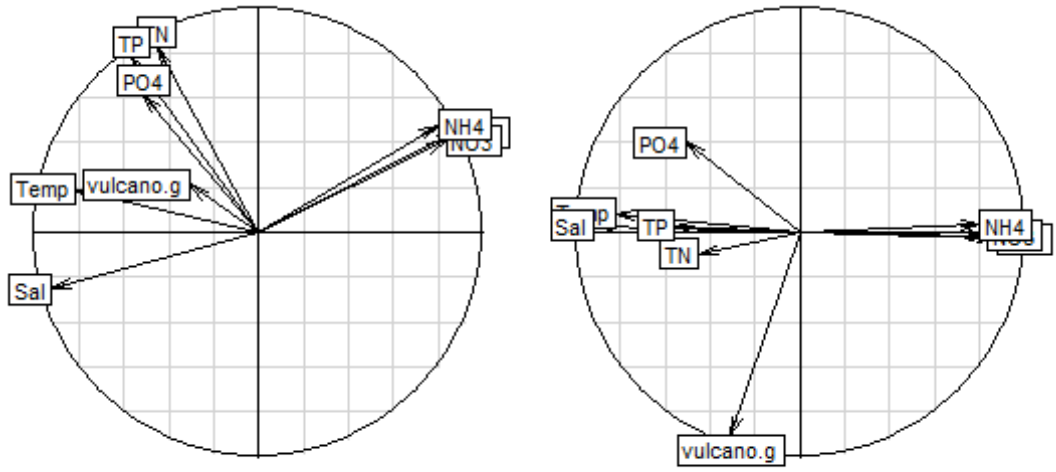


Figure 9: Principal Component Analysis for *Vulcanodinium rugosum* abundance in water column (Vulcano.L) related to all environmental factors (Temp: temperature, Sal: salinity, TP: total phosphorus, TN: total nitrogen, PO_4: phosphorus, NO_2: nitrite, NO_3: nitrate) (A: axis 1-2 & B: axis 1-3).

A B

Figure 10: Principal Component Analysis for *Vulcanodinium rugosum* abundance in water column related to water temperature and salinity (A:axis 1-2 and B: axis 1-3) – projection of *V. rugosum* abundance in water column (numbers are the sampling months).



A B

Figure 11: Principal Component Analyses (PCA) applied to *Vulcanodinium rugosum* abundance on macrophytes (Vulcano.g) in relation to environmental factors (Temp: temperature, Sal: salinity, TN: total nitrogen, TP: total phosphorus, NO_2: nitrate, NO_3: nitrite, PO_4: phosphorus (A: axis 1-2 & B: axis 1-3).

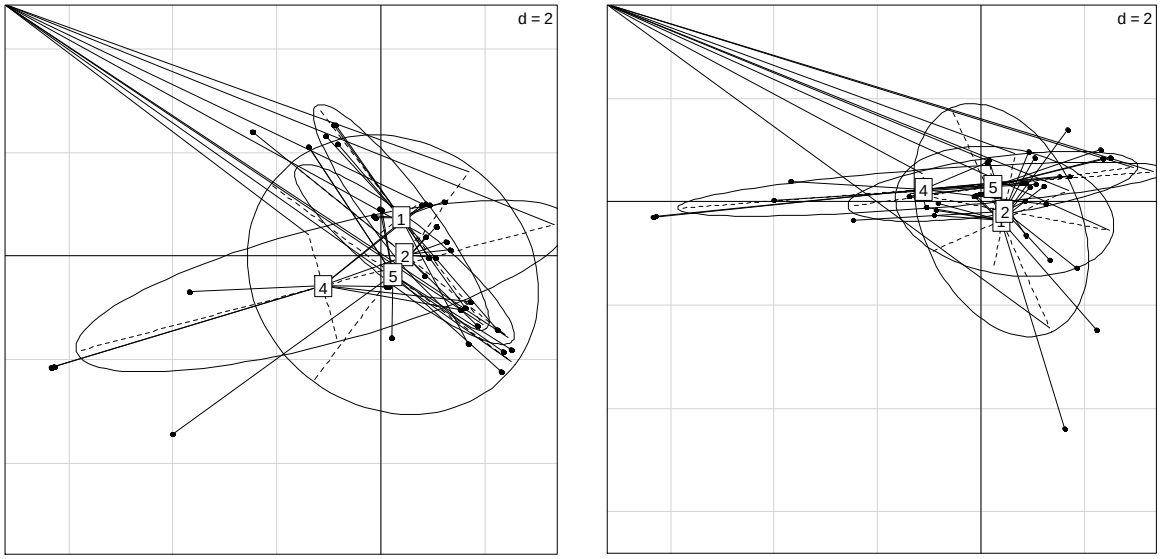


Figure 12: Principal Component Analyses (PCA) for *Vulcanodinium rugosum* concentrations on macrophytes related to environmental factors (A:axis 1-2 and B: axis 1-3) – projection of *V. rugosum* concentration (numbers are the sampling months).