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Optimization of sampling, cell collection and counting for the monitoring of benthic harmful algal blooms: application to *Ostreopsis* spp. blooms in the Mediterranean Sea

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ABSTRACT

Toxic blooms of *Ostreopsis* spp. are greatly challenging to monitor due to the complexity and variability of cell repartition among benthic and pelagic compartments. This results in marked differences in employed methodologies for the survey of their dynamics and hampers the definition of the associated toxic risk. The present study aims at testing and improving common methodologies used for sampling, processing and counting of field samples. It contributes to the identification of the most suitable strategies for the monitoring and mitigation of *Ostreopsis* blooms in coastal waters. For a sampling based on the collection of macrophytes, the role of the fixative addition and of agitation steps in the efficiency of epiphytic cell collection was defined. For planktonic estimations, the influence of the volume used for concentrating water samples was characterized as a function of *Ostreopsis* cell abundance. The deployment of artificial substrates was tested and confirmed strong advantages of this new sampling methodology, including an integration of a part of the spatial and temporal variability of the cell distribution.

Key words: Patchiness, macrophyte substrate, artificial substrate, recommendations for monitoring,

1. Introduction

Toxic dinoflagellate species of the genus *Ostreopsis* are distributed worldwide, from tropical to temperate areas, and are particularly widespread along the coasts of the Mediterranean Sea (Accoroni and Totti 2016; Mangialajo et al. 2011; Parsons et al. 2012). Their toxicity is associated with the synthesis of palytoxin-like compounds that include isobaric palytoxin, ovatoxins (Uchida et al. 2013; Brissard et al. 2014; Garcia-Altares et al. 2015) and mascarenotoxins (Rossi et al. 2010; Scalco et al. 2012). Intense blooms of *Ostreopsis* were related to noxious effects on human health, including skin irritations and respiratory disorder observed in swimmers and beach-goers (Del Favero et al. 2012; Tichadou et al. 2010; Vila et al. 2016). Deleterious effects of *Ostreopsis* blooms were also reported on marine invertebrates (Guidi-Guilvard et al. 2012; Pagliara and Caroppo 2012; Gorbi et al. 2013). To date, however, the knowledge of the ecology and ecotoxicology associated with *Ostreopsis* blooms is still scanty. Active compounds that can have deleterious effects have not been fully identified and/or characterized yet (Ciminiello et al. 2014; Vila et al. 2016). Epidemiology studies can provide crucial information about the link between *Ostreopsis* cell abundances and their toxic impacts. When considering *Ostreopsis* blooms, this approach shows some strong limitations, however. Primarily, clinical symptoms associated with exposure to *Ostreopsis* are non-specific and can be easily misinterpreted (Vila et al. 2016). Secondly, estimates of *Ostreopsis* cell density are intrinsically challenging because the distribution of cells during blooms is highly complex and variable, fluctuating between both benthic and pelagic compartments (Accoroni and Totti 2016). This type of distribution poses major problems for the formulation of optimized sampling protocols and for the assessment of the relationship between abundance and toxicity.

Cells of *Ostreopsis* spp. are predominantly benthic, epiphytic on macroalgae but also located on hard substrates such as rocks, sand or mollusk shells (Faust 1995; Parsons et al.

2012; Totti et al. 2010; Vila et al. 2001). On the seabed, *Ostreopsis* cells, like other benthic microalgae, are extremely patchy in distribution (GEOHAB 2012). This patchiness probably reflects small-scale variations in habitat characteristics, such as differences in exposure to turbulence or variety of substrates (Parsons and Preskitt 2007; Totti et al. 2010). Cells of *Ostreopsis* spp. are also commonly observed in the water column (Vila *et al*. 2001; Mangialajo et al. 2011), as free-living cells or agglutinated in mucilaginous aggregates that can float in the water column or accumulate at the sea surface. Field observations during *Ostreopsis* cf. *ovata* Fukuyo blooms in the Bay of Villefranche-sur-mer (France) showed that mucilaginous aggregates often accumulate at the air-water interface during the daylight, and especially in the afternoon (Jauzein and Lemée, pers. observation), suggesting the existence of a diel rhythm in vertical migrations of aggregates (Vila et al. 2008).

Ostreopsis mucilage seems to play a central role in the vertical distribution of cells. On the bottom, mucous filaments allow for the attachment of cells to surfaces (Honsell et al. 2013). For resuspended cells, extracellular mucilage can increase the buoyancy of cells by reducing the density of algal particles (Reynolds 2007). The mucilage matrix is also probably responsible for diel vertical migrations of dense aggregates of *Ostreopsis* cells in the water column. Migrations of aggregates, moving upward with sunrise and downward at sunset, have already been described for benthic microorganisms, including a mixture of diatoms, cyanobacteria and dinoflagellates, by Faust and Gulledge (1996). They are probably driven by the formation of gas bubbles that are trapped in the mucous matrix and regulate the buoyancy of mucilaginous aggregates. As specifically described for benthic cyanobacteria (World Health Organization, 2003) and diatoms (Fernández-Méndez et al. 2014), the diel formation of such gas bubbles can be defined from the balance between oxygen production by photosynthesis and consumption by respiration.

The complex and variable repartition of *Ostreopsis* cells, from the bottom to the water column, greatly complicates the design and implementation of quantitative sampling strategies for a suitable monitoring of *Ostreopsis* blooms and other BHABs (Giussani et al. 2017). This has resulted in marked differences in methodologies currently employed and reported in the literature, applied to the study of ecology and population dynamics of benthic toxic dinoflagellates. The need for reaching a consensus regarding protocols employed for the sampling, treatment and analyses of BHABs is stressed so that meaningful comparisons can be made among studies (GEOHAB 2012). It also opens perspectives for new technologies to be developed and validated (e.g. Tester et al. 2014; Jauzein et al. 2016; Mangialajo et al. 2017; Vassalli et al. 2018) in order to improve the precision and representativeness of data collection. The aim of the present study was to test, improve and validate major current protocols used for estimation of abundances of benthic harmful dinoflagellates, with a specific focus on *Ostreopsis* spp. In the framework of the EU project M3-HABs (http://m3-habs.net/), several techniques were compared, including sampling strategies (in particular the test of artificial substrate deployment), isolation techniques for epiphytic microalgae, and counting procedures. The present work allows for going further on the definition of the most suitable strategies for the monitoring and mitigation of BHABs.

2. Material and methods

2.1. Study sites

Dynamics of *Ostreopsis* blooms were monitored in six different coastal zones of the Mediterranean Sea. Two sites were located in the French Mediterranean coast (Villefranche-sur-mer and Nice); the others were located in Tunisia (Salammbô), Lebanon (Batroun) and Italy (Quarto and Ancona) (Figure 1). All these sites are characterized by sheltered rocky

coasts and similar dominant macroalgal communities (Table 1). The *Ostreopsis* species detected in the study areas were *Ostreopsis* cf. *ovata* Fukuyo in coastal waters of France, Tunisia and Italy and *Ostreopsis fattorussoi* Accoroni, Romagnoli et Totti in Lebanon. The presence of *O.* cf. *ovata* was also recorded in Lebanese coastal waters, but was almost negligible (Casabianca and Penna, pers. comm.).

2.2. Optimization of sampling methods

In each study area, three stations, about 10 m apart from each other, were simultaneously monitored during intensive surveys and used to test three different sampling methods. First, concentrations of planktonic *Ostreopsis* cells in the water column were estimated 20 cm above the bottom, over a seabed covered by macroalgae and situated at about 50 cm depth. For this planktonic sampling, plastic bottles were filled with 250 mL of seawater and then capped under water. These samples were collected in the morning (between 8:00 and 10:00 am, local time), taking care to avoid mechanical resuspension of benthic cells during sampling that could have artificially increased planktonic concentrations.

Second, abundances of benthic cells of *Ostreopsis* were estimated from the collection of macrophytes. This benthic sampling was performed in the morning, at about 50 cm depth, targeting the most abundant macroalgal species of the site. For each benthic sample, between 5 and 15 g of fresh weight of macroalgae were carefully collected with the surrounding seawater, using a 250 mL plastic bottle that was capped under water.

The third sampling method was based on the deployment of artificial substrates that allowed for the collection of planktonic and resuspended *Ostreopsis* cells. Artificial substrates consisted of rectangular pieces (2.5 cm × 27 cm) of window screen (1.3 mm porosity) that were fixed on a rigid frame as described in Jauzein et al. (2016). Each frame was attached to a weight and a small subsurface float in order to maintain the device at about 50 cm depth. With

the fixation systems, artificial substrates were held tightly on both sides and naturally positioned perpendicularly to the water flow. Artificial substrates were deployed on the same stations where benthic and planktonic samples were collected. They were incubated for 24h, a period that ended when planktonic and benthic samples were taken. Pieces of artificial substrates were collected using scissors and plastic bottles of 250 mL, as described in Jauzein et al. (2016).

Right after sampling, all bottles were brought back to the laboratory in less than one hour and fixed with acidic Lugol's solution (1% final concentration). Planktonic samples were immediately stored at 4 °C in the dark until analysis and processed in less than a month. Before counting, sedimentation columns were used to concentrate planktonic samples according to the Utermöhl method (Utermöhl 1958). For each sample, a 50 mL subsample was left to settle for 24 h, then planktonic *Ostreopsis* cells were enumerated using an inverted microscope (Axiovert 40 Zeiss light microscope at 100x or 200x magnification).

Samples from biological and artificial substrates were treated similarly. After addition of the fixative, sampling bottles were vigorously shaken for 10 sec in order to dislodge epiphytic *Ostreopsis* cells, then substrates were rinsed two times with 100 mL of FSW (Filtered Sea Water) to optimize cell collection efficiency. Water collected after agitation and washing was passed through a 500 µm meshed filter and mixed. The total volume obtained for each sample was recorded. A 50 mL subsample was then taken and stored at 4 °C in the dark until processing. For macroalgal samples, the macroalgae put aside was wrung out and immediately weighted. *Ostreopsis* cells were enumerated using a 1 mL Sedgewick Rafter Counting Cell examined with light microscope (Axio Scope.A1 Zeiss microscope at 100x or 200x magnifications). Cell abundances on macroalgal samples were expressed as number of cells per gram of fresh weight of macroalgae (cells.g^{-1} fw), while abundances on artificial

substrates were expressed as cells per cm² according to Weisstein (2013) and Tester et al. (2014).

Data from all the three different sampling methods are here reported for the Bay of Villefranche-sur-mer site (France) for summers 2014 and 2015. Data from all the other sites, located in France, Italy, Tunisia and Lebanon, refer to summer 2015 and consist of benthic and artificial substrates data only.

During the *O*. cf. *ovata* bloom that occurred in the Bay of Villefranche-sur-mer (France) in 2015, an additional experiment was specifically set up for characterizing the efficiency of *Ostreopsis* cell collection using artificial substrates. For this purpose, several deployment durations were tested for the artificial substrates in the field. During this bloom event, three pieces of artificial substrates were incubated weekly in each of the three stations monitored in order to follow bloom dynamics. This number of pieces was drastically increased during an exceptional sampling day, with the deployment of twelve pieces of artificial substrates in two stations. For this experiment, three independent frames were set up in each station, holding four pieces of artificial substrates each. One piece of substrate was carefully collected on each frame after 4h, 8h, 12h and 24h of deployment, using 250 mL plastic bottles. Samples were treated as described above.

2.2. Optimization of the detachment of epiphytic microalgal cells from macroalgae

In the Bay of Villefranche-sur-mer (France), additional samples of macrophytes were collected during the *O*. cf. *ovata* blooms in 2014 and 2015 in order to test and optimize methods for cell detachment. Each year, two sets of sampling were done: one under low level (< 20,000 cells.g^{-1} fw) and one under high level (> 100,000 cells.g^{-1} fw) of epiphytic *O*. cf. *ovata* abundances. Each time, fifteen benthic samples of the same macroalgal species (either *Halopteris scoparia* or *Dictyota* spp. or *Laurencia* spp.) were taken in the same surface (1 m²)

of sea bottom, at about 50 cm depth. Sampling was performed using plastic bottles that were filled with 5 to 20 g fw of macroalgal thallus and ~250 mL of surrounding seawater, and then capped under water. This specific sampling was used to analyze the role of the fixative addition and of the washing steps in the efficiency of the separation between macroalgal substrate and epiphytic *Ostreopsis* cells.

Three conditions were tested during the processing of these benthic samples (Figure 2): the separation step was performed without fixative addition (only seawater) or after addition of either acidic Lugol's solution or non-acidic Lugol's solution at 1% final concentration. Five replicates were analyzed per condition. For each individual sample, several sub-samples (10 mL) were collected during the successive detachment procedures. They allowed for estimations of the resuspended microalgal cell concentration *(i)* before the agitation of the benthic sample, *(ii)* after 10 sec of agitation of the benthic sample, *(iii)* at the end of the 1st washing step (after 10 sec of agitation of the macroalgal sample in 100 mL of FSW), *(iv)* at the end of the 2nd washing step, and *(v)* in the final volume combining water collected after agitation and washing of the benthic sample (Figure 2). Every sub-samples of the series collected without fixative addition were then spiked with 100 µL of acidic Lugol's solution (1% final concentration) for their preservation. All sub-samples were kept at 4 °C in the dark until analysis. Replicated cell counts of *O*. cf. *ovata* cells were done using a 1 mL Sedgewick Rafter Counting Cell and light microscope (Axio Scope.A1 Zeiss microscope). They allowed for estimations of the contribution of each step (from agitation to washing of the macroalgal substrate) in the detachment and collection of epiphytic *Ostreopsis* cells.

2.3. Optimization of counting techniques

Tests were run in order to estimate the accuracy of the counting of planktonic *Otreopsis* cells using the Utermöhl method. Surveys done in the Bay of Villefranche-sur-mer

(France) in 2014 and 2015 allowed for the collection of 78 planktonic samples during the development and decline of *O.* cf. *ovata* blooms. For all these samples, a 50 mL aliquot was settled in a sedimentation chamber for 24h before enumeration of *Ostreopsis* cells on an inverted microscope, as described above. For 38 of the available samples, showing cell concentrations ranging from 140 cells.L^{-1} to 14,000 cells.L^{-1}, additional measurements were done from two additional aliquots: one aliquot of 10 mL was simultaneously settled for 4h, and another aliquot of 100 mL was settled for 48h. Fourteen samples collected from artificial substrates during the monitoring of the *O.* cf. *ovata* blooms were also treated similarly, in order to increase the number of estimations at high concentration levels. Estimates of *Ostreopsis* cell concentrations obtained after sedimentation of 10 mL (settled 4h), 50 mL (settled 24h) or 100 mL (settled 48h) were compared. All the sedimentation chambers (HydroBios©) used had the same diameter but varied in height for defining volumes of 10 mL, 50 mL or 100 mL.

2.4. Statistical analyses

Linear regression analyses were used to compare different sampling and counting procedures, such as planktonic *versus* benthic sampling or artificial substrates *versus* benthic sampling, as well as for comparing various volumes of sedimentation of planktonic samples. The level of significance for regression slopes was set at $p < 0.05$. When testing conditions helping for the detachment of *Ostreopsis* epiphytic cells from macroalgae, analyses of variances were performed to identify and characterize the influence of significant factors on data distribution. A three-way ANOVA was used to determine the influence of the level of abundance (low or high), the year of survey, and the type of fixative addition on the proportion of cells detached from the macroalgal substrate before and after successive agitation steps. For each step, a second set of analysis was run after pooling the data as a

function of the factors highlighted as significant in the three-way ANOVA. Before agitation of benthic samples, data obtained under low *versus* high levels of abundance were normally distributed and Student's t-test was used to compare their means. Additional analyses of variance were conducted on data sets obtained during the processing of benthic samples, to determine the impact of the type of fixative addition on the detachment of epiphytic cells. When variances were homogeneous, data were analyzed by one-way ANOVA and Fisher's Least Significant Difference (LSD) test was performed to determine pairwise differences of means between data groups; data series of the washing steps followed these assumptions. For data series obtained after 10 sec of agitation, variances were not homogeneous and were analyzed by the non-parametric Kruskal-Wallis test and Bonferroni test. Finally, for testing the efficiency of the Utermöhl method for counting planktonic *Ostreopsis* cells, cell abundances obtained after settlement of 10 or 100 mL were standardized by the level of abundance obtained for 50 mL settlement and expressed as percentages. The distribution of these relative cell abundances was compared to the fixed value of 100% by one-sample t-tests in order to define if these values were significantly different from the reference counts (obtained for 50 mL samples). All the statistical analyses were performed using the Statgraphics® Centurion 18 software.

3. Results

3.1. Optimization of sampling methods

The intense surveys of *Ostreopsis* cf. *ovata* blooms that occurred in the Bay of Villefranche-sur-mer (France) in 2014 and 2015 allowed for the collection of samples under various levels of *O*. cf. *ovata* cell abundance. Those blooms lasted from mid-June to the end of July. Maximal abundances of 1.29×10^6 and 1.35×10^6 cells.g^{-1} fw were recorded in the

benthic communities in mid-July, in 2014 and 2015, respectively. When data were expressed as means between the three monitoring stations and log transformed, planktonic concentrations of *O*. cf. *ovata* cells were positively correlated with benthic abundances and characterized by the linear regression model $Y = 0.555\ X$ ($r^2 = 0.73$, $p < 0.01$). Planktonic concentrations in this data set were estimated from 50 mL aliquots of seawater. A detailed analysis of the data showed that the associated methodology was not sensitive enough to allow for precise measurements all along the density gradient tested, however. When considering a representation of the data for each station, the pattern of distribution of planktonic concentrations as a function of benthic ones showed a deviation from a linear correlation model: under low levels of abundance, several series of points clustered as a function of planktonic concentration levels, when some zero values were recorded (Figure 3A). This low precision of planktonic counts was noted for benthic concentrations lower than 13,500 cells.g^{-1} fw. Using the linear regression model defined above, this threshold corresponded to a planktonic concentration of 196 cells.L^{-1}, that is about 200 cells.L^{-1}.

Abundances of *Ostreopsis* cells collected from artificial substrates showed a positive correlation with benthic abundances. This relationship was observed when data were expressed per station in Villefranche Bay (Figure 3B) or as mean values between stations across all study sites (Figure 3C), and without any apparent drift in data distribution. When combining mean values obtained from the six different coastal zones of the Mediterranean Sea that were monitored, the final data set was modeled by $Y = 0.641\ X + 2.346$ ($r^2 = 0.55$, $p < 0.01$), where the abundance collected from artificial substrates (Y, in cell.100 cm^{-2}) is expressed as a function of benthic abundance (X, in cells.g^{-1} fw). This data set also fitted well with the equation defined by Tester et al. (2014) (Figure 3C); this other linear regression model was parameterized from data that were in a different range of abundances (between 0

and 1,300 cells.g^{-1} fw) than the one of the present study (between 1,100 and 813,000 cells.g^{-1} fw).

In order to estimate the efficiency of sampling using artificial substrates in detail, different deployment durations were tested in the field, in the Bay of Villefranche-sur-mer. Similar results were obtained from the two stations where the experiment was conducted. Variations of abundance of collected *Ostreopsis* cells showed a saturating kinetic as a function of time (Figure 4): abundances increased almost linearly with time during the first few hours, then the cell recruitment rate slowed down until the abundance reached a plateau. With the set-up of artificial substrates used in the present study, the concentration of cells collected over 8h and 12h exceeded 60 and 80% of the maximal abundances recorded after 24h, respectively.

3.2. Optimization of the detachment of microalgal epiphytic cells from macroalgae

Four sets of experiments were conducted in the Bay of Villefranche-sur-mer (France), in order to define the role of the fixative addition and the efficiency of the washing steps in the detachment of epiphytic *Ostreopsis* cells from their macroalgal substrate. Two sets were conducted per year, targeting low *versus* high levels of abundance. The addition of fixative before the separation step did not show a significant effect on the total number of isolated *O.* cf. *ovata* cells (Figure 5A). When pooling the data from the different detachment procedures for each set of the experiment, high levels of abundance were characterized by average values (n = 15) of 15.8×10^4 ($\pm$ 0.6×10^4) cells.g^{-1} fw and 24.7×10^4 ($\pm$ 9.0×10^4) cells.g^{-1} fw in 2014 and 2015, respectively, while low levels of abundance were defined from respective average values of 1.2×10^4 ($\pm$ 0.6×10^4) cells.g^{-1} fw and 0.6×10^4 ($\pm$ 0.3×10^4) cells.g^{-1} fw. A strong variability was noted among replicated samples (Figure 5A): a factor up to 4 was observed

between the minimum and the maximum benthic concentrations that were estimated on the same macroalgal species and in the same 1 m² of seabed.

A three-way ANOVA was performed to determine the influence of three factors (the low or high level of abundance, the year of survey, and the type of fixative addition) on the proportion of *O.* cf. *ovata* cells detached from the macroalgal substrate during the processing of benthic samples (Figure 2, Table 2). According to the results obtained, the quantity of isolated *O*. cf. *ovata* cells before agitation of the sample was significantly affected by the level of abundance and the year of survey ($p < 0.001$), while the type of fixative addition did not show a significant effect (Table 2). Conversely, after 10 sec of agitation of the macroalgal substrate in the surrounding seawater or during each of the washing steps, the type of fixative addition had a significant effect on the proportion of isolated microalgal cells ($p < 0.001$), while neither the level of abundance nor the year of survey had a significant influence (Table 2).

When focusing on data obtained before agitation of benthic samples, the level of abundance of epiphytic *O*. cf. *ovata* cells had a significant effect on the proportion of detached cells (Figure 5B): for the two years of survey, the proportion of cells loosely attached to the substrate was lower when the benthic concentration was high (t-test, $n = 15$, $p < 0.001$ for 2014 data and $p < 0.005$ for 2015 data). Thus, the agitation steps had a higher contribution to the collection of epiphytic microalgal cells under high levels of benthic abundance.

Data obtained after carrying out each agitation step (conducted either in the surrounding seawater or during washings) were pooled as a function of the only significant factor highlighted: the type of fixative addition ($n = 20$, Figure 5C). No significant difference was observed if acidic Lugol's solution was used instead of non-acidic Lugol's solution. Results showed that 10 sec of agitation of the macroalgal substrate allowed for the collection

of a significantly higher number of *O*. cf. *ovata* cells if the fixative was added before the separation step (Figure 5C). Conversely, the contribution of each washing step in epiphytic cell detachment was significantly higher for samples processed in seawater than for samples fixed with Lugol's solution (Figure 5C). The proportion of cells isolated during the two washing steps reached 40 (±18) % when the separation step was done in seawater; this proportion was 13 (±7) % if the procedures were done after addition of Lugol's solution. Specifically, when benthic samples were immediately fixed with Lugol's solution, the second washing step allowed for recovering only 2.9 (± 2.3) % of the total number of epiphytic *O*. cf. *ovata* cells from benthic samples.

A potential correlation between the percentage of isolated cells and the weight of macroalgal substrate was tested for each agitation step (in the surrounding seawater or during washings) and each type of fixative addition (n = 20). These analyses were performed to test if a maximal weight of macroalgae can be defined for 250 mL sampling bottles, over which microalgal epiphytic cells could not be dislodged efficiently due to the high volume of macroalgal biomass. Some trends were apparent: for every series of samples (with or without fixative addition), the percentage of cells isolated after 10 sec of agitation in the surrounding water tended to decrease with increasing macroalgal weight. Conversely, the percentage of cells isolated after each washing step tended to be positively correlated with the weight of macroalgae. However, when using linear regression models to simulate these data sets, none of these trends was significant (r^2 comprised between 0.01 and 0.23 and $p > 0.05$). Thus, in the present study, the quantity of biomass of macroalgae (between 5 and 20 g fw) was not defined as a potential factor of variation in the proportion of isolated benthic cells.

3.3. Optimization of counting techniques

A series of counts of *O.* cf. *ovata* planktonic cells was performed using different sedimentation volumes of either 10, 50 or 100 mL. For these planktonic samples, the comparison of data sets showed significant positive correlations between cell counts done using a 10 mL column, or a 100 mL column, and the reference counts done with the 50 mL column. Respective linear models were described by $Y = 0.975\ X$ ($r^2 = 0.98$, $p < 0.001$) and $Y = 0.889\ X$ ($r^2 = 0.99$, $p < 0.001$) for the 10 mL and the 100 mL counts, when expressed as a function of the 50 mL counts. In comparison to the regression model $Y = X$, the slopes obtained suggested a potential underestimation of cell concentration when using a 100 mL column, instead of a 50 mL column.

In order to characterize these relationships in more details, values obtained after sedimentation of 10 or 100 mL were expressed as percentages, relatively to 50 mL counts. This allowed for running a standardization of the data sets according to the overall level of concentration and to analyze the sensitivity of the method regarding low-density levels. When doing so, a threshold of 3,000 cells.L^{-1} could be defined, under which the variability of estimations increased when the level of abundance decreased (Figure 6A, Figure 6B, Figure 6C). This increase in variability was described in details with coefficients of variation (CV), analyzing their dependence on the minimal level of abundance fixed for the data set. For both series of standardized data (10 mL and 100 mL), CV was stable over the gradient of cell concentration tested, on condition that planktonic concentrations were higher than 3,000 cells.L^{-1} (Figure 6C). Interestingly, increasing the number of data taken into account in CV estimations by adding values of abundance lower than this threshold increased the dispersion of data distribution (Figure 6C). The threshold of 3,000 cells.L^{-1} corresponded to 150 cells counted in 50 mL. This means that counting less than 150 cells per slide decreased the precision of the measurements performed using the Utermöhl method. Current results also showed that counting cells after sedimentation of 10 mL was surprisingly efficient. Indeed,

for most of the samples, using a 10 mL sedimentation column instead of 100 mL column only induced a slight increase in measurement variability (Figure 6C). A strong difference in signal variability was noted between the two series of data when planktonic concentrations were lower than 1,000 cells.L^{-1}; this concentration corresponded to less than 10 cells counted in 10 mL.

When considering only values for which the level of precision of the reference counts was optimal (planktonic concentrations higher than 3,000 cells.L^{-1}), relative abundances from 10 mL and 100 mL counts were normally distributed and could be compared to the fixed value of 100% that was representative of the 50 mL counts. Data analysis showed no significant difference between cell counts performed using either a 10 mL column or a 50 mL column (relative data were not significantly different from 100%, one-sample t-test, $n = 27$, $p = 0.88$) (Figure 6D). However, results clearly confirmed that counting *O*. cf. *ovata* cells using a 100 mL sedimentation column induced a significant underestimation of planktonic concentrations (percentages were significantly lower than 100%, one-sample t-test, $n = 27$, $p < 0.001$) (Figure 6D). This underestimation corresponded to approximately 10% of the optimal cell counts.

4. DISCUSSION

4.1. Monitoring of the benthic stock

The majority of field surveys that aim at understanding the ecology of *Ostreopsis* spp. target the benthic pool, quantifying epiphytic *Ostreopsis* cells by collection of macrophytes (Okolodkov et al. 2007; Shears and Ross 2009; Carnicer et al. 2015). This sampling choice is motivated by the fact that benthic populations of *Ostreopsis* represent the main stock of cells during bloom events. In the frame of alert systems, this is also a reason why Mangialajo et al.

(2011) recommended to policy makers and managers to primarily monitor benthic abundances: these estimations of the benthic pool are considered more conservative and more representative of bloom dynamics than planktonic concentrations. Additionally, for the mitigation of BHABs, the processing of benthic samples (from sampling to data analysis) does not require more than few hours, a fundamental characteristic for a timely alert in a management perspective.

This macrophyte-based collection method shows some important limitations and disadvantages, however. First, the replicability of cell quantification is strongly affected by the fact that epiphytic *Ostreopsis* cells are extremely patchy in distribution (GEOHAB 2012). This patchiness may be partly explained by a preference that *Ostreopsis* cells can show for some particular macroalgal species, groups or morphologies (Parsons and Preskitt 2007; Totti et al. 2010). It could also come from small-scale variations in micro-hydrodynamics conditions. In the present study, such a patchy distribution was evident for *O*. cf. *ovata*, when comparing levels of abundance estimated for different sets of benthic samples that were collected in the same area of seabed and on the same macroalgae species: a factor up to 4 was observed between the minimum and the maximum concentrations for a single set of replicated samples. Strong patchiness poses a major problem for design and execution of macrophyte-based sampling (GEOHAB 2012). It makes it necessary to have a high level of replication per site in order to obtain reliable data, representative of benthic population abundance.

The sampling of benthic microalgal populations based on the collection of macrophytes has two other inherent problems. One relates to habitat disruption and may become problematic in case of intense survey. Another is associated with the required standardization of data sets. Benthic microalgal cell abundances can be normalized either per unit of surface area or of fresh or dry weight of macrophyte substrate (e.g. Totti et al. 2010). For ecological studies, a normalization of benthic abundances to a known surface area is the

only one allowing for a comparison with estimations done from hard substrates. However, the 3D morphology of macroalgae often makes the estimation of surface area complex and challenging. Thus, most of the recent ecological studies have continued to report benthic abundances expressed per unit of weight of macrophytes (e.g. Selina et al. 2014; Carnicer et al. 2015). This diversity in potential standardization of benthic abundances can limit the making of meaningful comparisons among studies.

Finally, another issue in the precision and reliability of benthic measurements results from the diversity of methodologies that are currently employed, and reported in previous studies, for the processing of macrophyte samples. One of the goals of the present study was to optimize the treatment of macrophyte samples for the monitoring of BHABs, focusing on the separation step between epiphytic *Ostreopsis* cells and their biotic substrates. According to previous reports on *Ostreopsis* blooms, macrophyte samples are always shaken in ambient seawater to dislodge epiphytic microalgal cells. However, this separation step is sometimes performed without any fixative addition (e.g. Totti et al. 2010) or after fixation of samples with either addition of acidic Lugol's solution (Cohu et al. 2011) or non-acidic Lugol's solution (e.g. Mangialajo et al. 2008), or formaldehyde (e.g. Okolodkov et al. 2007). Similarly, benthic cell counts are sometimes performed on the water collected after a single agitation step (e.g. Shears and Ross 2009), whereas other studies report measurements performed after rinsing the macrophyte substrate twice (e.g. Cohu et al. 2011) or even more than three times (e.g. Totti et al. 2010). The present study allows for a clarification of the potential role of fixative addition, agitation and washing in the efficiency of the separation step in order to go further in the definition of standardized protocols.

Present results showed that the proportion of *Ostreopsis* cells loosely attached to biotic substrates was lower when the benthic cell abundances were high. This suggests that the formation of dense mucilaginous mats, where *Ostreopsis* cells are embedded, makes epiphytic

cells harder to detach and to be resuspended. In terms of survey, this also indicates that the more the bloom develops, the more the agitation steps become crucial for the collection of epiphytic *Ostreopsis* cells. Furthermore, according to present results, the addition of fixative (either acidic or non-acidic Lugol's solution) does favor the detachment of *Ostreopsis* cells from their biotic substrates during the agitation steps. In fact, in fixed benthic samples, 97% of *Ostreopsis* cells are detached and collected after the completion of an agitation step and a single washing of the macroalgal substrate, whatever the level of benthic concentration is. This proportion appears satisfactory in terms of collection efficiency. Without addition of Lugol's solution, a second washing of the substrate is mandatory: it provides the collection of still about 15% of the attached cells' pool. In the end, when two successive washing steps are completed, levels of benthic abundances estimated with or without initial addition of Lugol's solution are not significantly different. Thus, these successive agitation steps compensate for the poor detachment efficiency of benthic *Ostreopsis* cells in seawater. When identifying suitable strategies in the frame of alert systems, it is important to limit as much as possible time-consuming tasks. In this case, collected macrophyte samples should be immediately fixed with Lugol's solution and the separation step should include a unique washing of the macrophyte substrate. For ecological studies, researchers can choose to either fix or not fix collected macrophyte samples before the separation step, knowing that similar estimations of benthic abundances of *Ostreopsis* are obtained when macrophyte material is rinsed twice.

4.2. Monitoring of the planktonic cell abundances

During bloom development and maintenance, numerous cells of *Ostreopsis* are commonly observed in the water column, while the main stock of the population is located on the seabed (Vila et al. 2001; Vila et al. 2016; Mangialajo et al. 2017). Part of these planktonic cells are considered as free-living and resuspended. In terms of survey, the presence of

Ostreopsis cells in the water column provides the opportunity to follow bloom dynamics from the direct sampling of seawater in the water column. This sampling choice can be motivated by the fact that, as observed in present results and reported in other field studies (Vila et al. 2001; Aligizaki and Nikolaidis 2006; Mangialajo et al. 2011, Asnaghi et al. 2012), planktonic concentrations of *Ostreopsis* cells showed a positive correlation with benthic abundances. Monitoring the planktonic compartment is interesting because it shows some important advantages: *(i)* the sampling is easy to perform, *(ii)* is not destructive, and *(iii)* allows for a partial integration of the complexity of the spatial distribution of *Ostreopsis* cells (in particular regarding the benthic patchiness). In the frame of alert systems, the planktonic fraction is also thought to be more directly related to the level of risk for human health than the benthic pool. Indeed, acute health disorders that were reported in the presence of *Ostreopsis* blooms mainly came from direct contact with seawater (skin irritations) and/or to toxic marine aerosols (respiratory irritations) (Vila et al. 2016).

Planktonic sampling for the monitoring of BHABs shows some limitations, however. A first one comes from the signal variability as a function of time, commonly higher for planktonic cell abundances than benthic ones. One main factor of this variability is linked with turbulence. Sudden turbulent conditions due to wave action can induce a resuspension of epiphytic *O*. cf. *ovata* cells (Accoroni and Totti 2016; Mangialajo et al. 2017). This can lead to abrupt changes in planktonic cell abundances that are not representative of bloom dynamics (Mangialajo et al. 2011; Vila et al. 2008). Furthermore, planktonic concentrations recorded at a fixed depth can also vary due to changes in the vertical distribution of mucilaginous aggregates. In an optimized sampling protocol, diel variations in cell repartition make it necessary to perform a rigorous sampling of *Ostreopsis* cells in the water column at a fixed depth and a fixed time of the day.

The present study highlights other potential issues associated with the precision of planktonic estimations, but also defines ways to avoid them using an appropriate protocol. During the survey of *Ostreopsis* blooms in the Bay of Villefranche-sur-mer (France), all samples from the planktonic fraction were counted using a 50 mL sedimentation chamber. This sampling and counting strategy showed a lower sensitivity than other tested procedures based on the collection of substrates. Such condition can be seen as a limitation for its usefulness in risk assessment of BHAB effects, in particular for early detection of BHAB development and anticipation of the toxic risk (Landsberg et al. 2005). The low sensitivity of planktonic estimations can be improved, however, by settling a higher volume of sample, typically of 100 mL instead of 50 mL. Under low levels of abundance, increasing the sedimentation volume allows for increasing the number of enumerated cells, and so the precision of the measurement.

In order to go further on the definition of an optimized protocol, the efficiency of counting using the Utermöhl method can be characterized by threshold values of abundance. According to present results, when using 50 mL chambers, estimations are only approximate for abundances lower than 200 cells.L^{-1} but have a maximal accuracy for abundances higher than 3,000 cells.L^{-1}. Thus, under low levels of *Ostreopsis* abundance (< 3,000 cells.L^{-1}), it is advisable to use a high volume of settlement, ideally of 100 mL, in order to optimize precision of estimations thanks to an increase in the number of enumerated cells. When concentrations are higher than the threshold of 3,000 cells.L^{-1}, abundances estimated using the Utermöhl method are significantly underestimated (10%) when using a 100 mL column, instead of a 10 to 50 mL chamber. This underestimation probably comes from an attachment of *Ostreopsis* cells to edges of columns during the sedimentation process, promoted by the cellular production of mucus and aggravated by elevated levels of cell density. High cylinders (≥ 100 mL) are often not recommended in monitoring programs because of the attachment of

organisms to the wall of columns and the formation of convection currents, which hinder cell sedimentation (Salmaso et al. 2017). A fixation of samples with formaldehyde might be a way to avoid this issue, but this was not tested here. In present results, addition of Lugol's solution was shown to help detachment of *Ostreopsis* cells from their substrate under agitation, but this effect did not appear efficient enough to avoid attachment of cells during gravity sedimentation. Thus, for moderate to high concentration of *Ostreopsis* cells in the seawater (> 3,000 cells.L^{-1}), optimal estimations of planktonic cell density are obtained settling no more than 50 mL of sample before counting.

In the frame of alert systems, the usefulness of planktonic survey is limited by the time required for the treatment of samples. Considering this issue, 100 mL column are not adapted to monitoring programs requiring fast feedback as they impose at least two days of settlement. Conversely, the present study points to the interest of using a 10 mL sedimentation column in order to shorten counting procedures: counting only ten or so cells in 10 mL already provide good estimations of planktonic concentrations, when densities can be estimated in a day (from sampling to counting). In the frame of ecological studies, precision of measurements is leading protocol choices, so higher volumes of sedimentation will be preferred.

4.3. Monitoring using the deployment of artificial substrates

As an alternative to the traditional macrophyte-based collection method or seawater sampling, a promising sampling strategy relies on the use of artificial substrates. Artificial substrates can be deployed in the field and allow for a concentration and easy collection of resuspended benthic cells (Tester et al. 2014). Several types of material have already been deployed as artificial substrates in the frame of BHAB monitoring, including test tube brushes and plastic plates (Bomber and Aikman, 1989) or nylon ropes (Faust, 2009). Parsons et al. (2017) recently tested the use of PVC tiles, burlap fabric and fiberglass window screens for

the monitoring of the benthic toxic dinoflagellate *Gambierdiscus* in the Florida Keys. In this last study, correlations reported between cell abundances collected on artificial substrates *versus* specific macrophyte species were weak, but similar to relationships obtained when comparing cell densities among different macrophyte hosts. Furthermore, during this survey, artificial substrates were retrieved one month after their deployment in the field; this long duration might have increased the variability of cell density of epiphytic populations. Indeed, recruitment and colonization of artificial substrates by benthic microalgal cells are direct consequences of immigration, disturbance and emigration of cells from the surrounding seawater on a short timescale (hours or days). Over weeks or months, other biological factors are also influencing the epiphytic microalgal community, including cell division, death or grazing (Dalu et al. 2014 and references herein). Biological interactions, such as allelopathy or competition, may also lead to a divergence of the epiphytic community over time that can make the pool of recruited cells less representative of the surrounding seawater (Tester et al. 2014).

Based on the above, Tester et al. (2014) recently reported a standardized method for the deployment of fiberglass screens for the monitoring of BHABs, based on short-term incubations (less than two days). Some optimizations of this latest methodology were recently described by Jauzein et al. (2016), including the definition of a new set-up and of an optimal mesh porosity of the substrates. In the present study, the efficiency of *Ostreopsis* cell collection using this optimized set-up, based on the deployment of screens, was characterized in more details.

Results from the present study corroborate correlations defined by Tester et al. (2014) between abundances of *Ostreopsis* cells collected on fiberglass screens and levels of benthic abundances. Tester et al. (2014) parameterized a linear regression model in a very low range of cell concentrations; *Ostreopsis* benthic abundances were comprised between 0 and 1,300

cells.g^{-1} fw. Interestingly, this model fitted well with data of the present study that consisted of moderate to high level of abundances (from 1,100 to 813,000 cells.g^{-1} fw). This comparison between studies confirms the good sensitivity of the sampling strategy based on the deployment of artificial substrates for the monitoring of *Ostreopsis* blooms. It is also interesting to note that the data set reported by Tester et al. (2014) pooled results from three different sites located in coastal waters of Belize and Malaysia, while the present study compiled results obtained from five different coastal zones of the Mediterranean Sea (Table 1). This shows that the correlation between abundances collected on artificial substrates and on macrophytes is highly robust, whatever the coastal zone is (and the associated physico-chemical conditions are), whoever the operator is and whatever the level of abundance is.

As discussed above, the sampling method based on the use of artificial substrates appears as well adapted to comparisons between sites and studies. This assumption can be justified by the fact that this methodology shows two main advantages: *(i)* collected abundances are not dependent on variations in composition and distribution of macrophytes in time and space and *(ii)* data can be easily standardized per unit of surface area (Tester et al. 2014). Another strength of this methodology relies on the time of incubation in the field that allows for an integrated estimation of the level of abundance in the surrounding seawater. Such integration combines a spatial dimension, that reduces the impact of the patchy benthic distribution, and a temporal dimension that can take into account diel migration of cells in the water column. In the end, cells collected on artificial substrates after few hours of deployment may provide a better representation of the BHAB population in a defined station than snapshots based on the sampling of few grams of macroalgae or few hundreds of mL of seawater.

Concerning potential disadvantages, main constrains linked with the use of artificial substrates are due to time-consuming tasks. Several hours of incubation are necessary for the

recruitment of resuspended benthic cells. In the frame of alert system, this limits the possibility of making quick estimations of abundances of BHABs that can be useful when the toxic risk is high. The incubation duration also requires a double visit to the sampling site, once to deploy the substrates and again to retrieve them (Tester et al., 2014).

The recent set-up defined by Jauzein et al. (2016) for the deployment of fiberglass screens in the field helps for limiting these disadvantages. In the first standardized protocol reported by Tester et al. (2014), pieces of fiberglass screens are attached to a rope and suspended freely in the water during the incubation. With this set-up, an accumulation of *Ostreopsis* cells on screens was observed during the first 24h, then densities of collected cells reached a plateau (Tester et al., 2014). Such a plateau, corresponding to an equilibrium between immigration and emigration rates, was also reached after 24h when screens were hold tight on both sides and maintained perpendicular to the water flow according to Jauzein et al. (2016). Both protocols lead to significant differences in cell recruitment rate, however, when considering time-scales lower than 24h. In the study of Tester et al. (2014), only 5% of the maximal abundance were collected after 12h of incubation. Present results show that this proportion reached 80% over 12h, and more than 60% over 8h, when using the modified set-up. With such an improved collection efficiency, artificial substrates can be considered an interesting tool not only for the monitoring of BHABs, but also for alert systems as they provide good estimations of population abundances in less than a day. On a broader point of view, the definition of the most suitable strategies for the monitoring and mitigation of BHABs would benefit from a comparison of this sampling strategy (based on the deployment of artificial substrates) with other recently defined methodologies, such as the use of a Benthic Dinoflagellate Integrator (BEDI) (Mangialajo et al. 2017).

5. Conclusion

Toxic blooms of *Ostreopsis* are challenging to monitor due to the complexity and variability of cell repartition among benthic and pelagic compartments. The present study makes a detailed comparison between methods currently employed for the survey of BHABs. It contributes to the identification of the most suitable strategies for the monitoring and alert setting for *Ostreopsis* blooms in coastal waters. For the treatment of macroalgal samples, an optimization of the separation step, allowing for the isolation of epiphytic cells, was defined: an early addition of Lugol increases the efficiency of *Ostreopsis* cell detachment and no more than two washings of the substrate are recommended. Planktonic surveys may be an interesting option for a quick estimation of the toxic risk but are not well adapted to monitoring purposes. The influence of the sedimentation volume used for concentrating water samples was characterized as a function of *Ostreopsis* cell abundance. Finally, the deployment of artificial substrates appears as a very promising sampling strategy for the monitoring of *Ostreopsis* blooms, in the frame of both ecological surveys and mitigation of BHABs. The strength of this new methodology is based on two main advantages: *(i)* an independence from the macroalgal substrate helping for comparison of data sets between sites and studies and *(ii)* an integration of the spatial and temporal variations of the *Ostreopsis* cell distribution during blooms. Such characterization of methods is a prerequisite for reaching a consensus regarding the definition of common protocols for the monitoring and mitigation of BHABs.

Acknowledgements

This work was supported by the European Union under the ENPI CBC Mediterranean Sea Basin Programme, within the project M3-HABs. Authors wish to thank Julie Rostan and

Cédric Darbon and Anna Maria Rossi for their help in field work and counting. French authors are part of the national French GDR PHYCOTOX (CNRS and Ifremer).

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LEGEND OF FIGURES

Figure 1. Map showing the locations of the six sites sampled for the monitoring of *Ostreopsis* blooms in the Mediterranean Sea.

Figure 2. Experimental procedures used for estimating the role of the fixative addition and of the washing steps in the efficiency of the separation between epiphytic *Ostreopsis* cells and macroalgal substrate. The number of replicated samples is indicated in italic. Four

sets of this experiment were conducted, under low *versus* high levels of benthic abundance of *Ostreopsis* cells, and during two successive years of survey.

Figure 3. Abundances of *Ostreopsis* cells estimated in the planktonic fraction (A) and collected from the deployment of artificial substrates (B and C), expressed as a function of benthic abundances. Data are plotted for each station (three per site) that were monitored in the Bay of Villefranche-sur-mer (France) (A and B) and as mean values between stations for each of the six sites of survey (C), located in France, Italy, Tunisia and Lebanon. The arrow shown in panel A corresponds to the threshold of 13,500 cells.g^{-1} fw. In the panel C, data are log-transformed and the linear regression model shown is the one reported by Tester et al. (2014) for the same genus, from surveys conducted in coastal areas of Belize and Malaysia. The square indicates the range of concentration values used by Tester et al. (2014) for data fitting.

Figure 4. Kinetics of *Ostreopsis* cells' accumulation on artificial substrates (window screens) deployed in the Bay of Villefranche-sur-mer (France) during summer 2015. Stations A and B are located on the same study site and are only 10 m apart from each other. Values correspond to mean abundances (± standard deviation) compiled from three replicate pieces of substrate that were hold on three different rigid frames. Data are expressed as percentages relatively to the mean level of abundance estimated for 24h deployment. Stars indicate missing data, when considering a regular increase in incubation time every 4h.

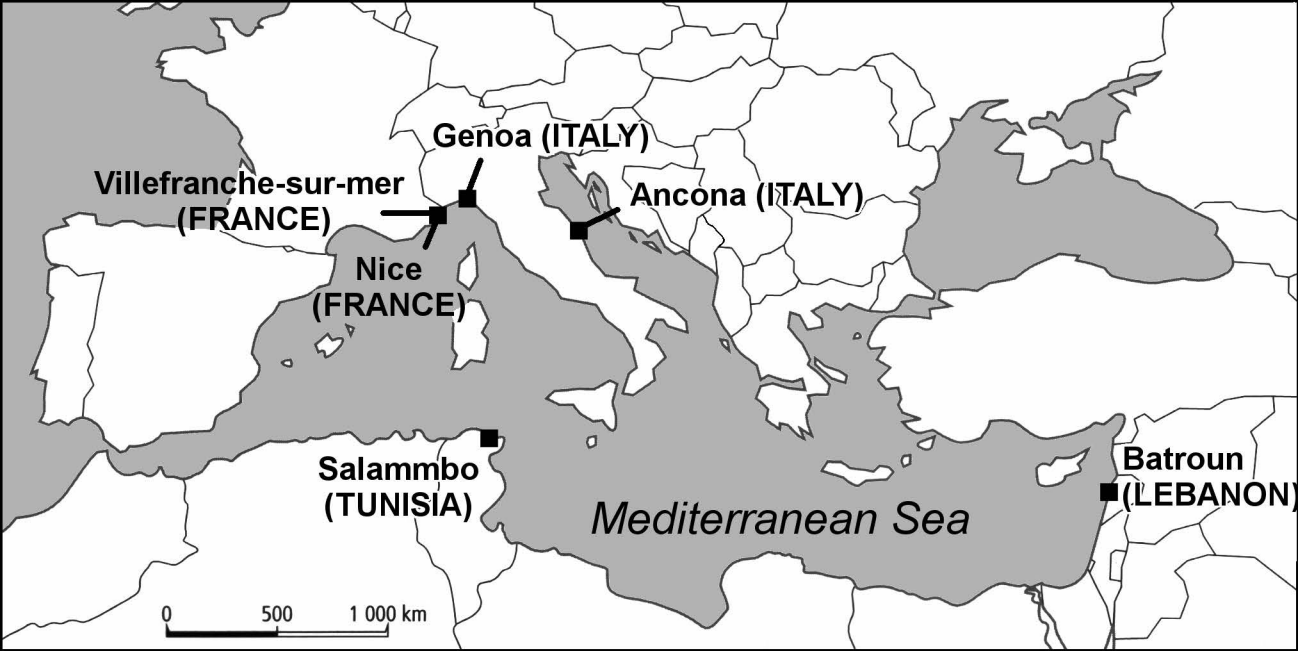
Figure 5. Contribution of fixative addition and of washing steps in the detachment of epiphytic *Ostreopsis* cells from their macroalgal substrate. Benthic abundances in the Bay of Villefranche-sur-mer (France), estimated after agitating the macroalgal substrate in the

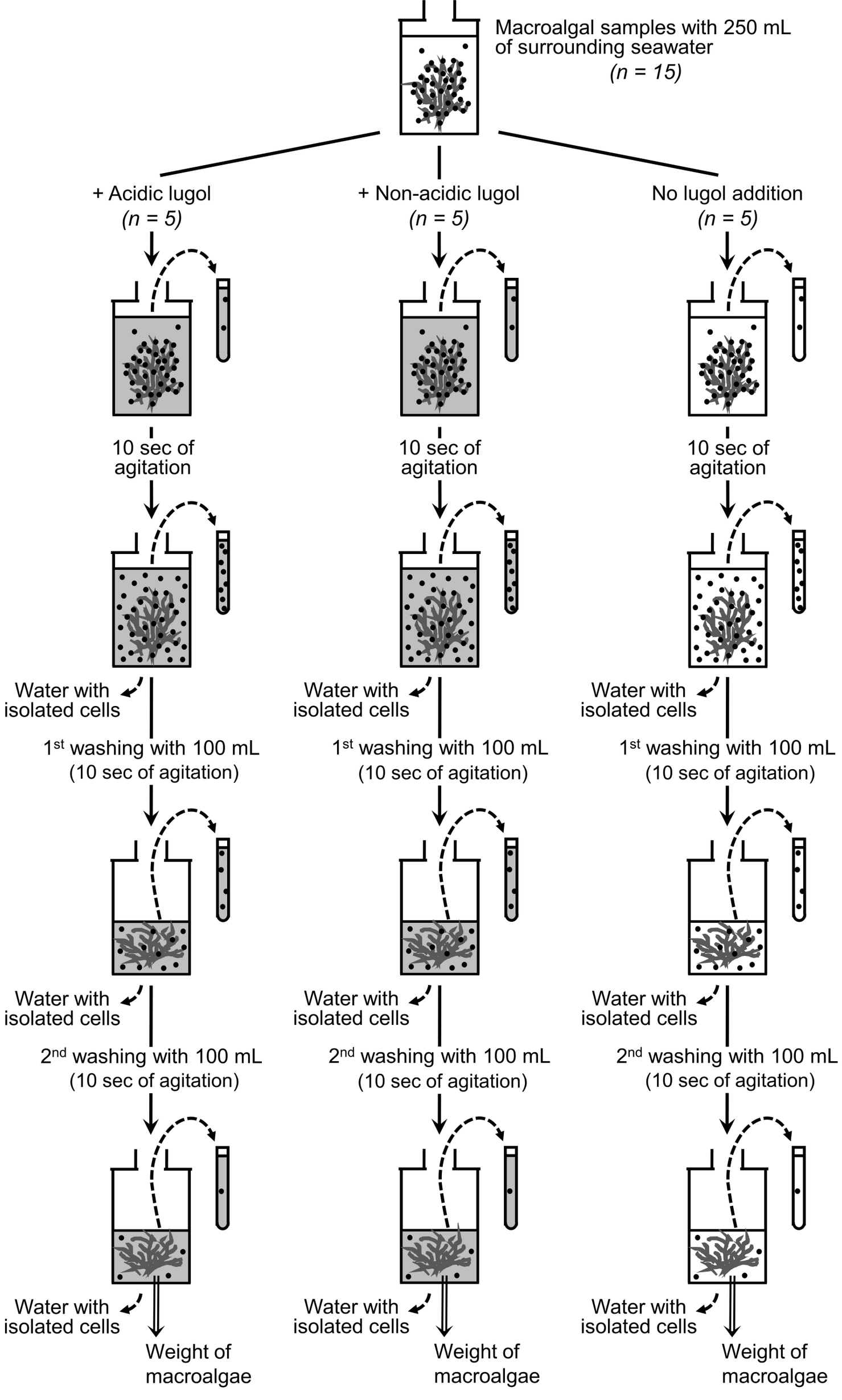
surrounding seawater and performing two successive washing steps, with or without initial addition of acidic Lugol's and non-acidic Lugol's solution (A). Details on the relative proportion of cells collected before (B) and after each of the agitation steps (C). Data plotted in panels B and C were pooled as a function of the significant factors identified from a three-way ANOVA. This ANOVA was used to determine the influence of the low or high level of abundance, the year of survey, and the type of fixative addition on the proportion of cells detached from the macroalgal substrate (Table 2). In the end, data correspond to average values (± standard deviation) and respective numbers of pooled samples are n = 5, n = 15 and n = 20 for panel A, B and C. Statistically significant differences are indicated by asterisks when a t-test was run (Panel B, ** $p < 0.01$, *** $p < 0.001$) and by letters when data were analyzed by either Kruskal-Wallis and Bonferroni tests or ANOVA and Fishers LSD tests (Panel C, $p < 0.001$).

Figure 6. Efficiency of counting planktonic concentration of *Ostreopsis* cells using the Utermöhl method. Estimations of *Ostreopsis* cell concentrations after settlement of 10 mL (A) and 100 mL (B) of seawater, reported as percentages of the associated counts done from 50 mL settlement. Comparison of these relative abundance data sets in terms of coefficient of variation, considered as a function of the number of cumulated samples and the range of abundance (C), and average values (± standard deviation) for data that were normally distributed (> 3,000 cells.L^{-1}, n = 27) (D). The dashed lines on panels A, B and C correspond to the level 100%, showing an equality between concentrations estimated using either a 10 mL or a 100 mL column of sedimentation, compared to the reference count done with a 50 mL column. On the panel D, results of statistical analyses comparing relative counts to the value 100% are shown (one-sample t-test, n = 27, *** $p < 0.001$).

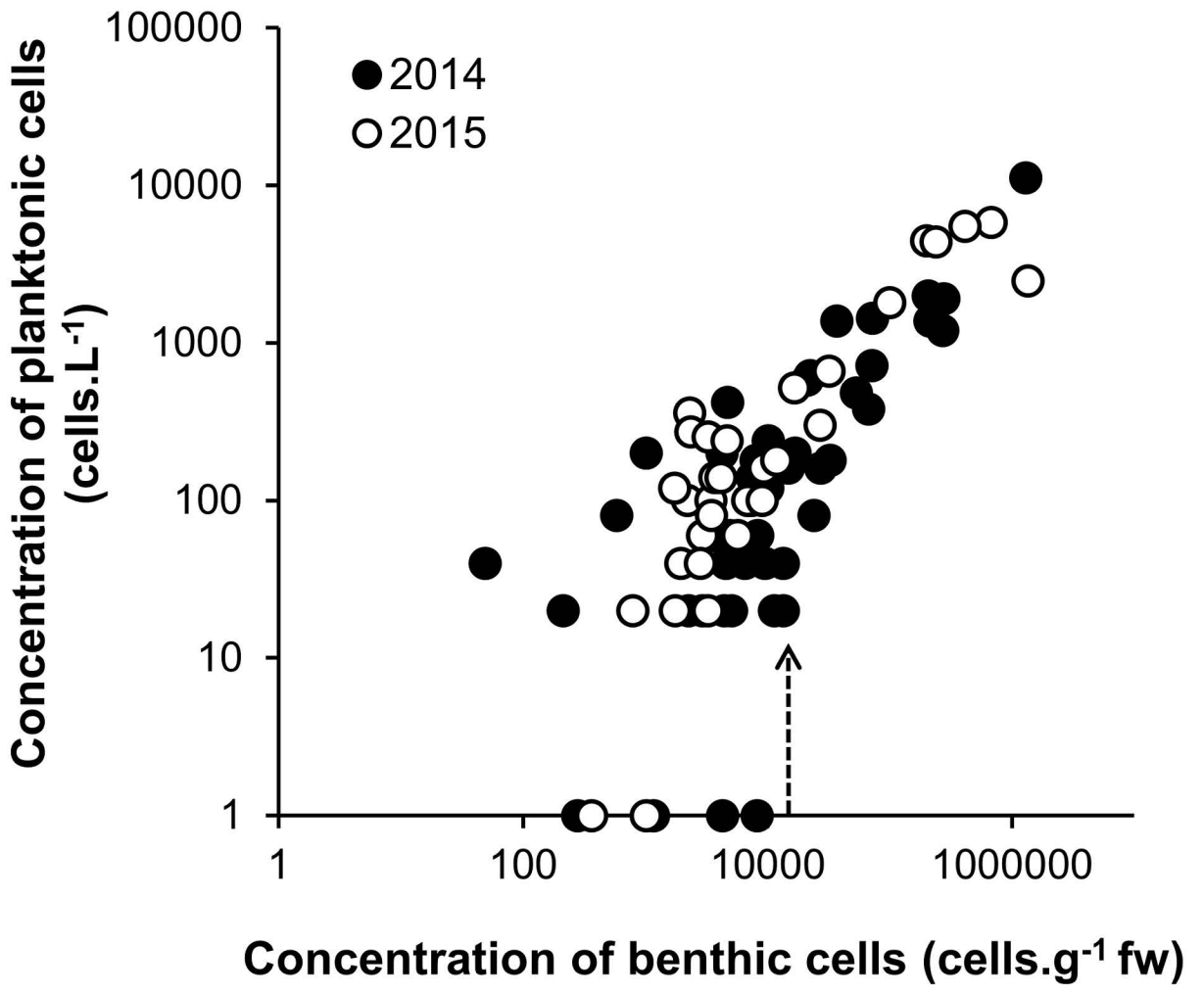
Table 1. Main characteristics of the study sites and *Ostreopsis* blooms that were monitored.

Table 2. Statistical analyses of data obtained when characterizing conditions helping for the detachment of epiphytic *Ostreopsis* cells from macroalgal substrate during the treatment of benthic samples. These results correspond to a three-way ANOVA used to determine the influence of three variables on the proportion of detached *O.* cf. *ovata* cells: the low or high level of benthic abundance, the year of survey (2014 or 2015), and the type of fixative addition (no addition, initial addition of acidic Lugol's solution, initial addition of non-acidic Lugol's solution). Four sets of data were analyzed: they correspond to abundances measured before the agitation of macroalgal samples, after 10 sec of agitation of the macroalgal samples and after one or two successive washing steps of the macroalgae with FSW.

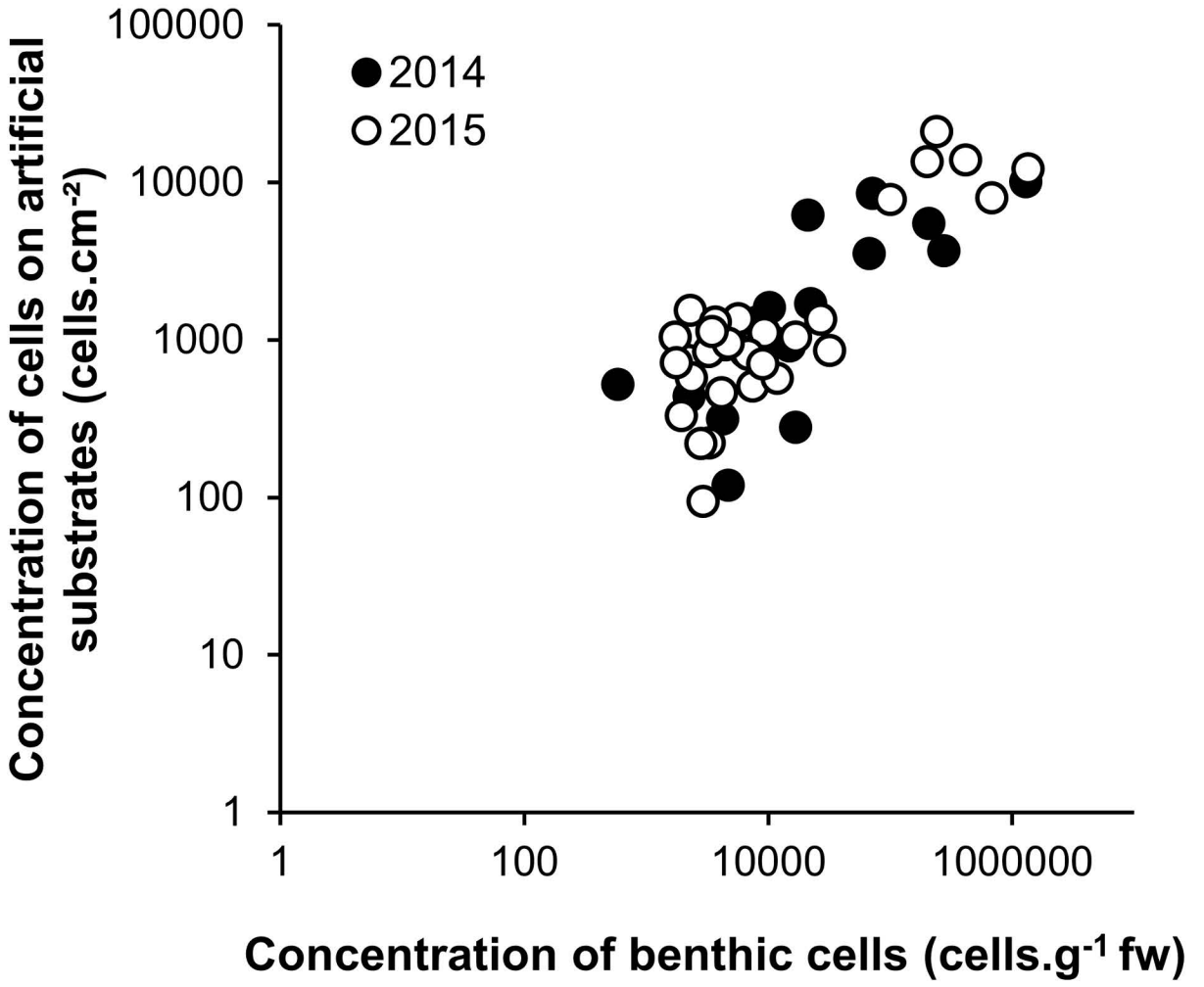

Genoa (ITALY)
Villefranche-sur-mer
(FRANCE)
Ancona (ITALY)
Nice
(FRANCE)
Salammbo
(TUNISIA)
Batroun
(LEBANON)
Mediterranean Sea
0
500
1 000 km


Macroalgal samples with 250 mL of surrounding seawater
(n = 15)
+ Acidic lugol
(n = 5)
+ Non-acidic lugol
(n = 5)
No lugol addition
(n = 5)
10 sec of agitation
10 sec of agitation
10 sec of agitation
Water with isolated cells
Water with isolated cells
Water with isolated cells
1st washing with 100 mL
(10 sec of agitation)
1st washing with 100 mL
(10 sec of agitation)
1st washing with 100 mL
(10 sec of agitation)
Water with isolated cells
Water with isolated cells
Water with isolated cells
2nd washing with 100 mL
(10 sec of agitation)
2nd washing with 100 mL
(10 sec of agitation)
2nd washing with 100 mL
(10 sec of agitation)
Water with isolated cells
Water with isolated cells
Water with isolated cells
Weight of macroalgae
Weight of macroalgae
Weight of macroalgae

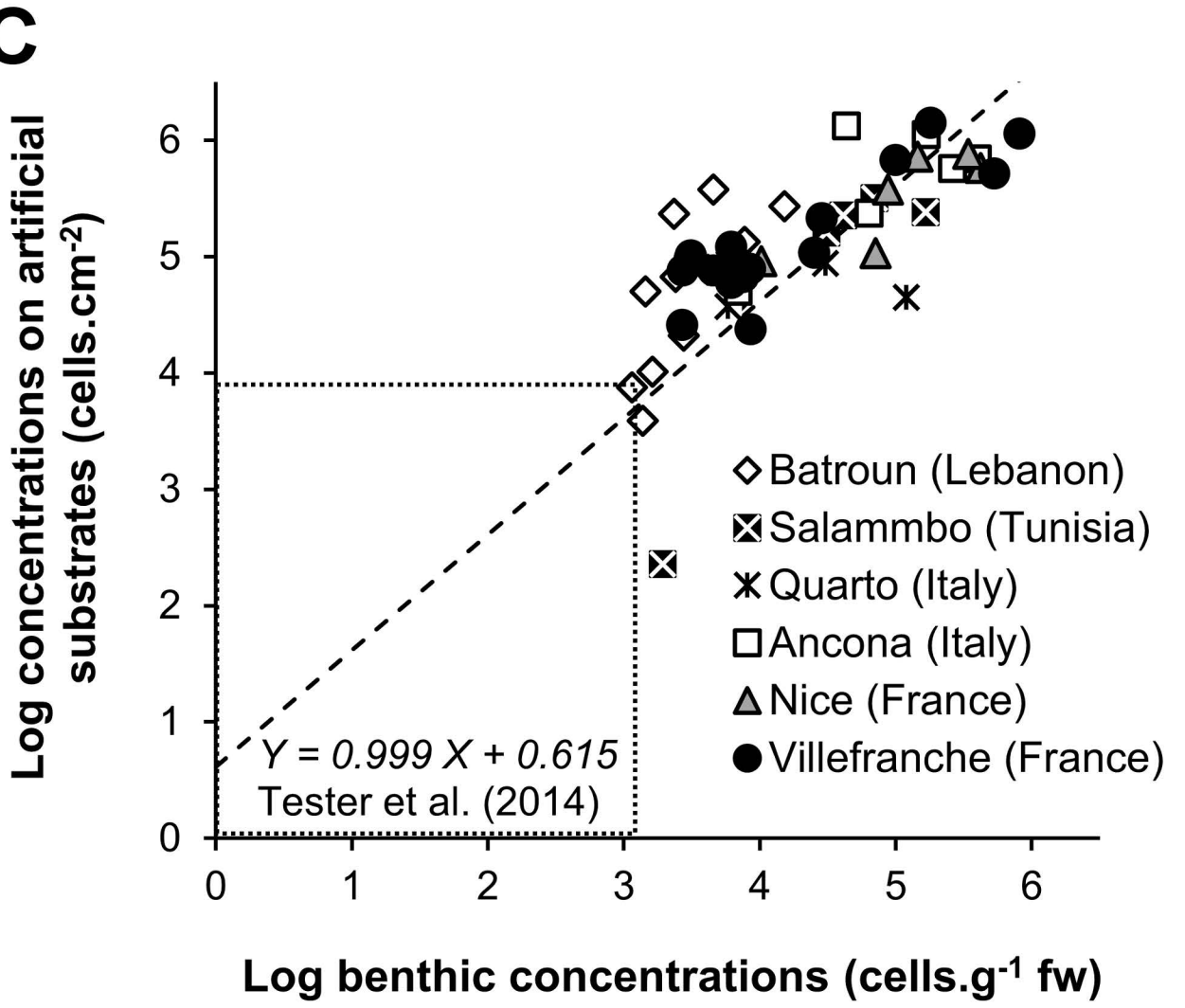
A

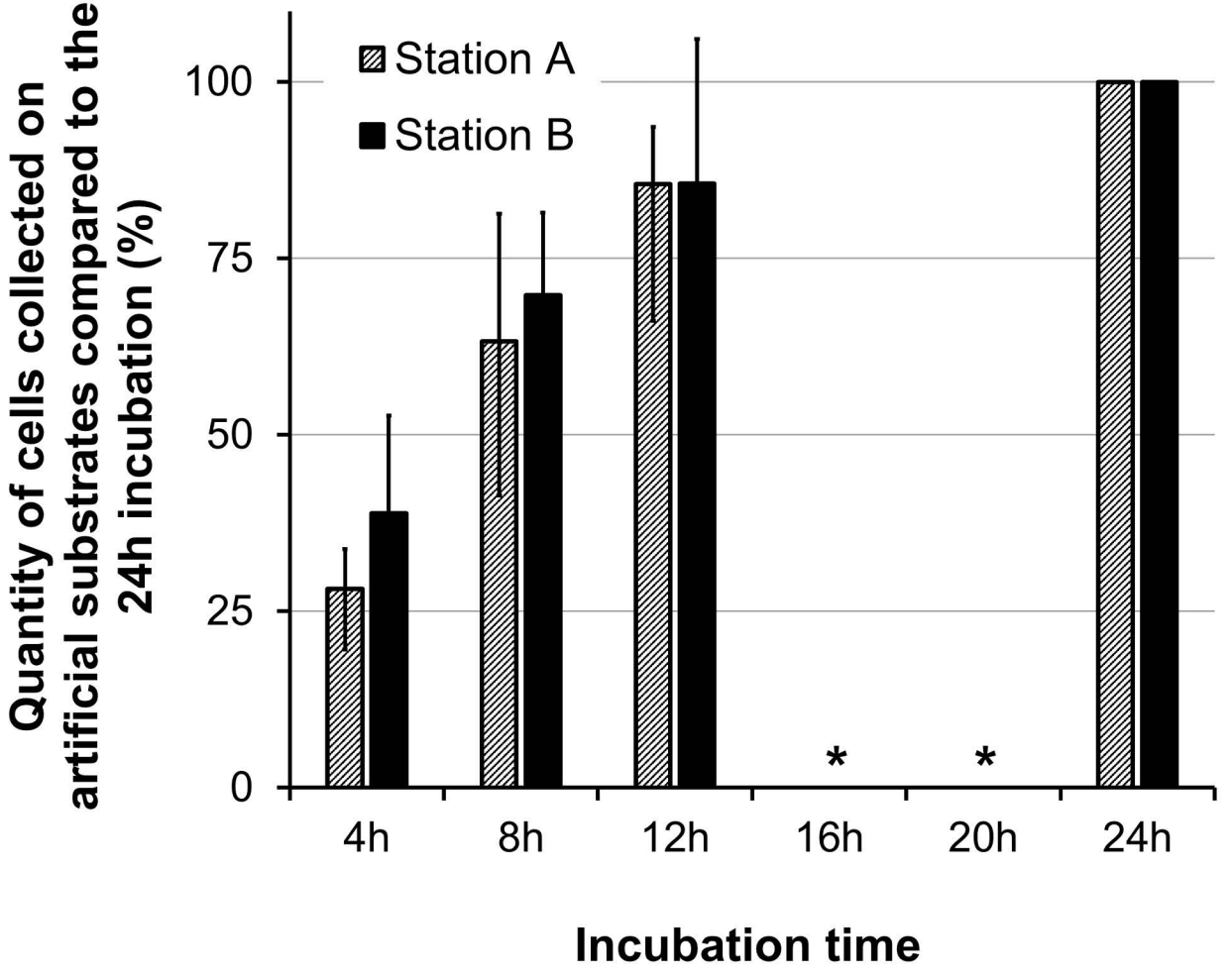

100000
10000
1000
100
10
1
2014
2015
Concentration of planktonic cells (cells.L-1)
1
100
10000
1000000
Concentration of benthic cells (cells.g-1 fw)

B

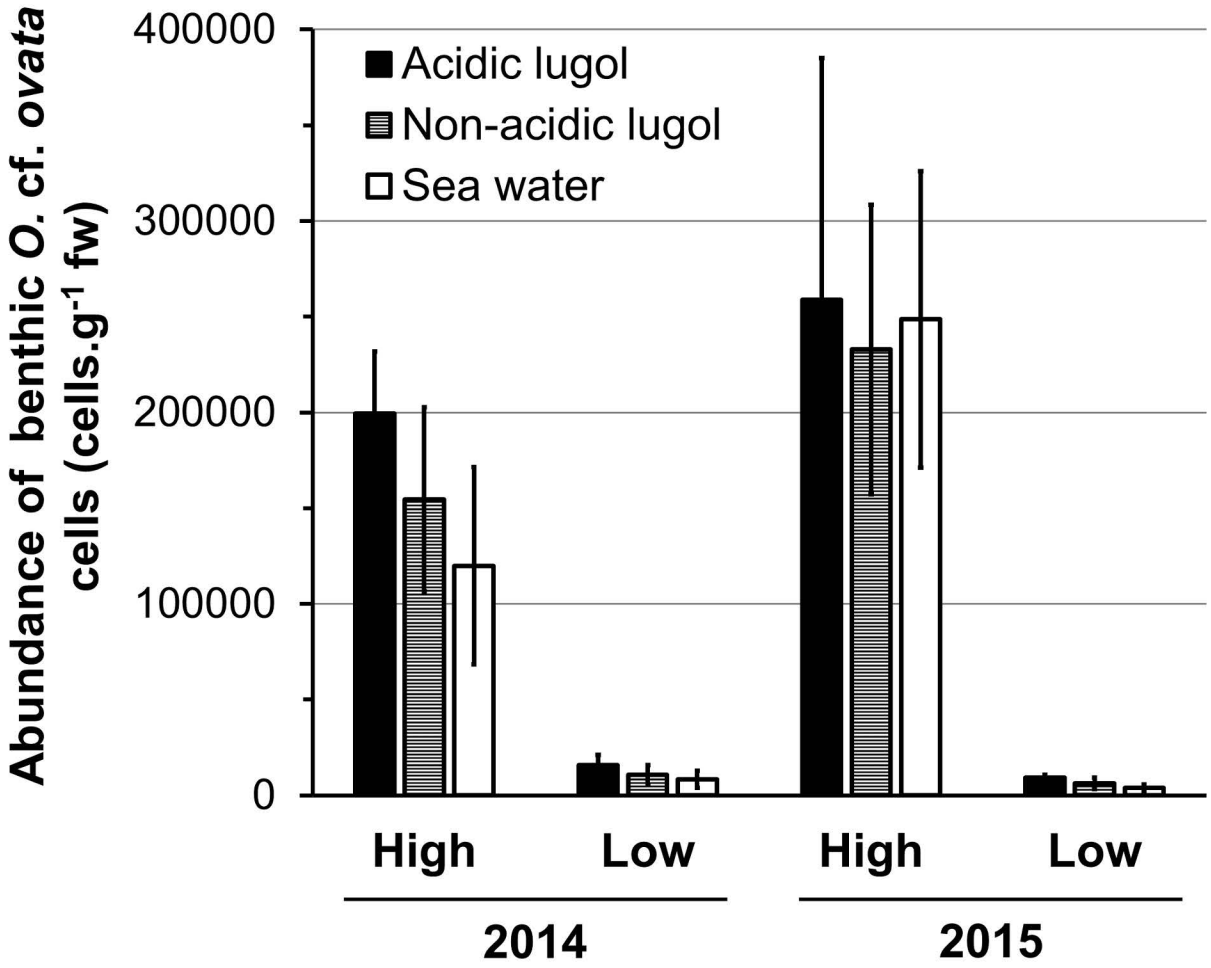

100000
10000
1000
100
10
1
2014
2015
Concentration of cells on artificial substrates (cells.cm-2)
1
100
10000
1000000
Concentration of benthic cells (cells.g-1 fw)

C

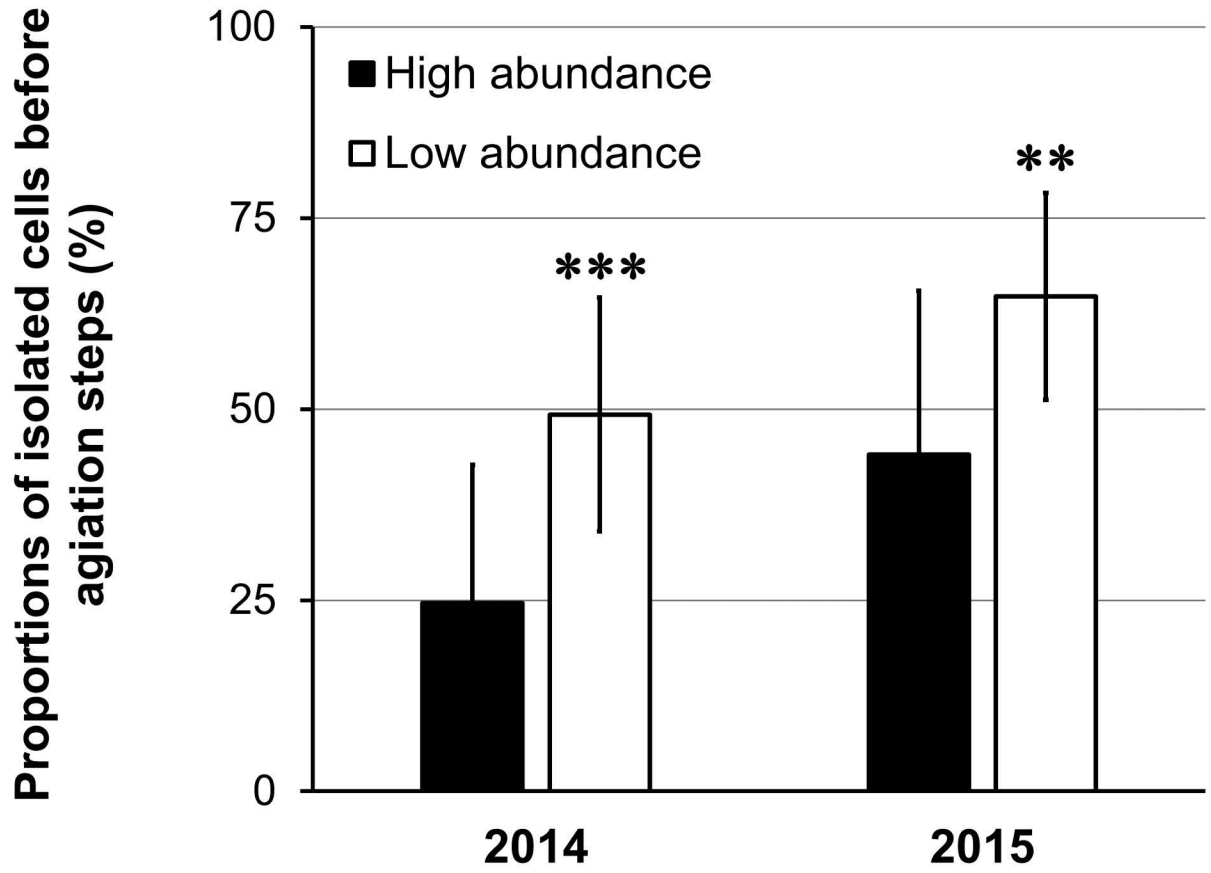

Log concentrations on artificial substrates (cells.cm-2)
0
1
2
3
4
5
6
Batroun (Lebanon)
Salammbo (Tunisia)
Quarto (Italy)
Ancona (Italy)
Nice (France)
Villefranche (France)
Y = 0.999 X + 0.615
Tester et al. (2014)
Log benthic concentrations (cells.g-1 fw)


Quantity of cells collected on artificial substrates compared to the 24h incubation (%)
Station A
Station B
100
75
50
25
0
*
*
4h
8h
12h
16h
20h
24h
Incubation time

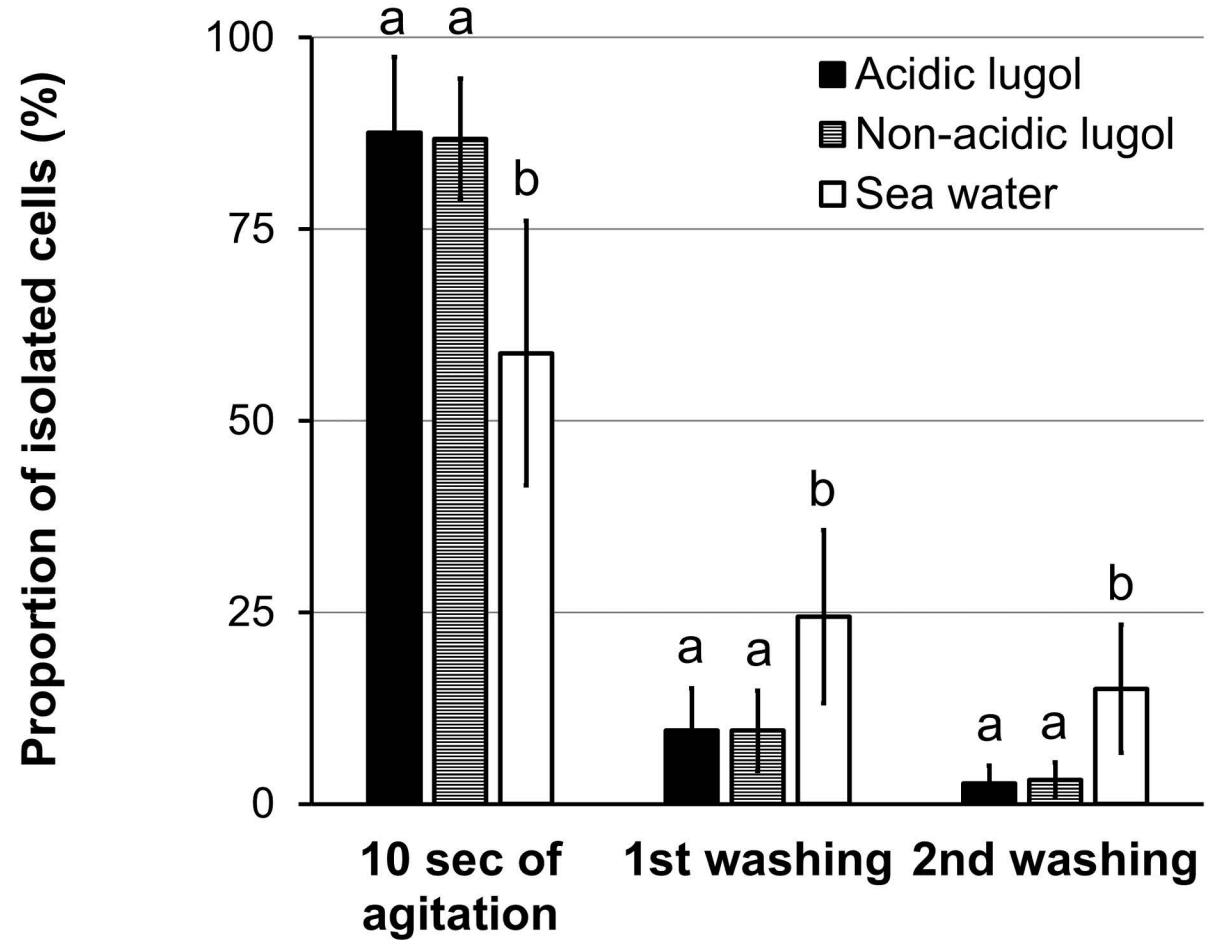
A


Abundance of benthic O. cf. ovata cells (cells.g-1 fw)
400000
300000
200000
100000
0
Acidic lugol
Non-acidic lugol
Sea water
High
Low
High
Low
2014
2015

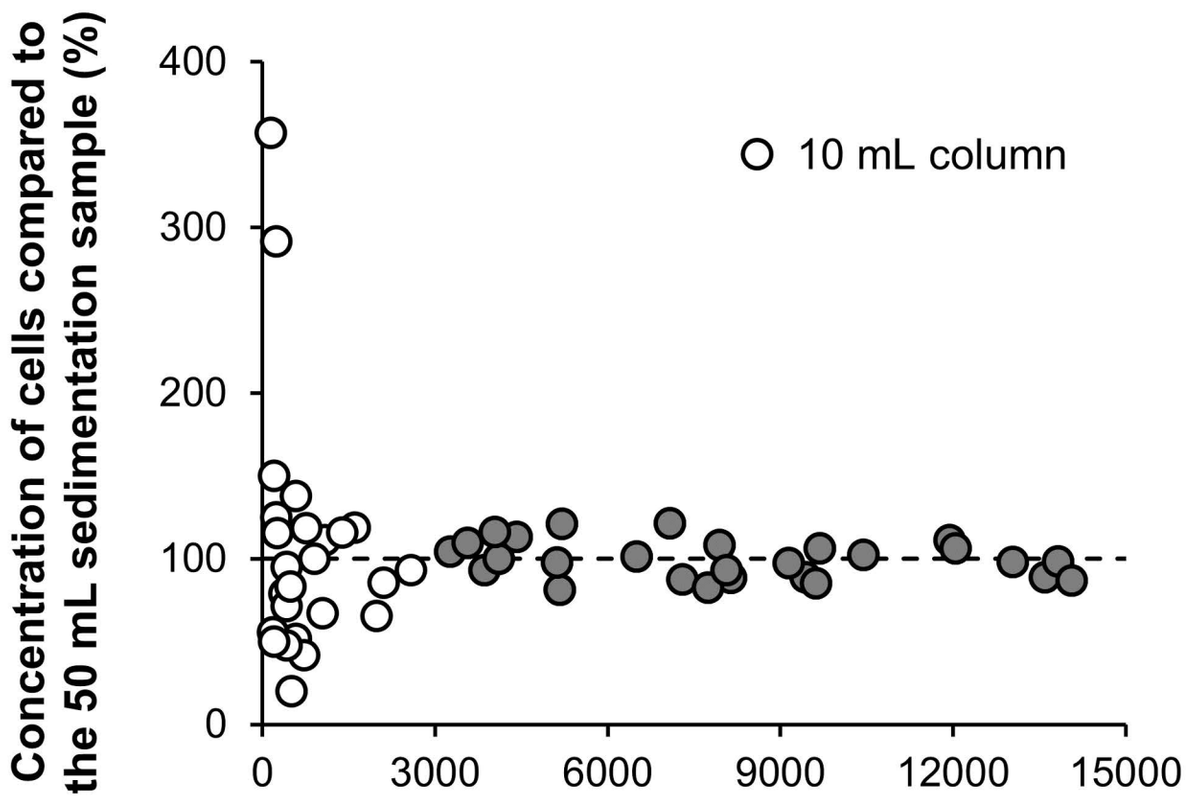
B


Proportions of isolated cells before agiation steps (%)
100
75
50
25
0
High abundance
Low abundance
***
**
2014
2015

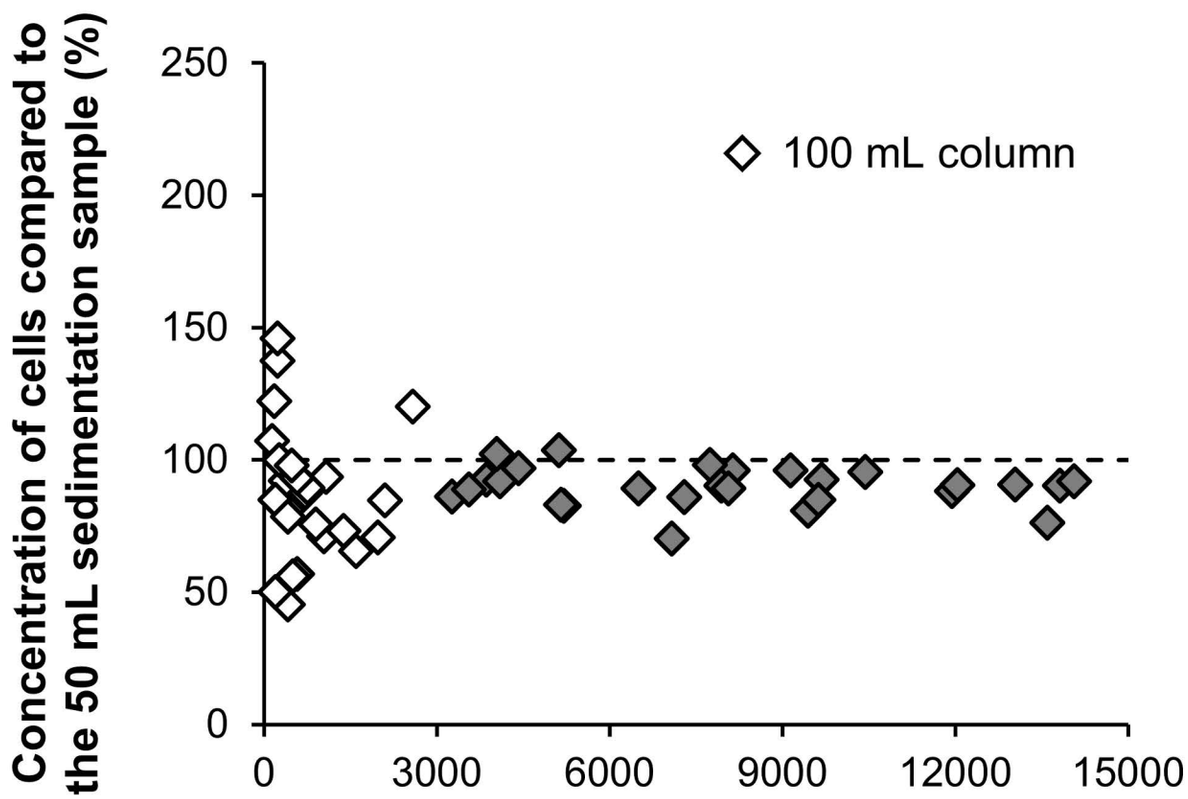
C


Proportion of isolated cells (%)
100
75
50
25
0
a a b
a a b
a a b
Acidic lugol
Non-acidic lugol
Sea water
10 sec of agitation
1st washing
2nd washing

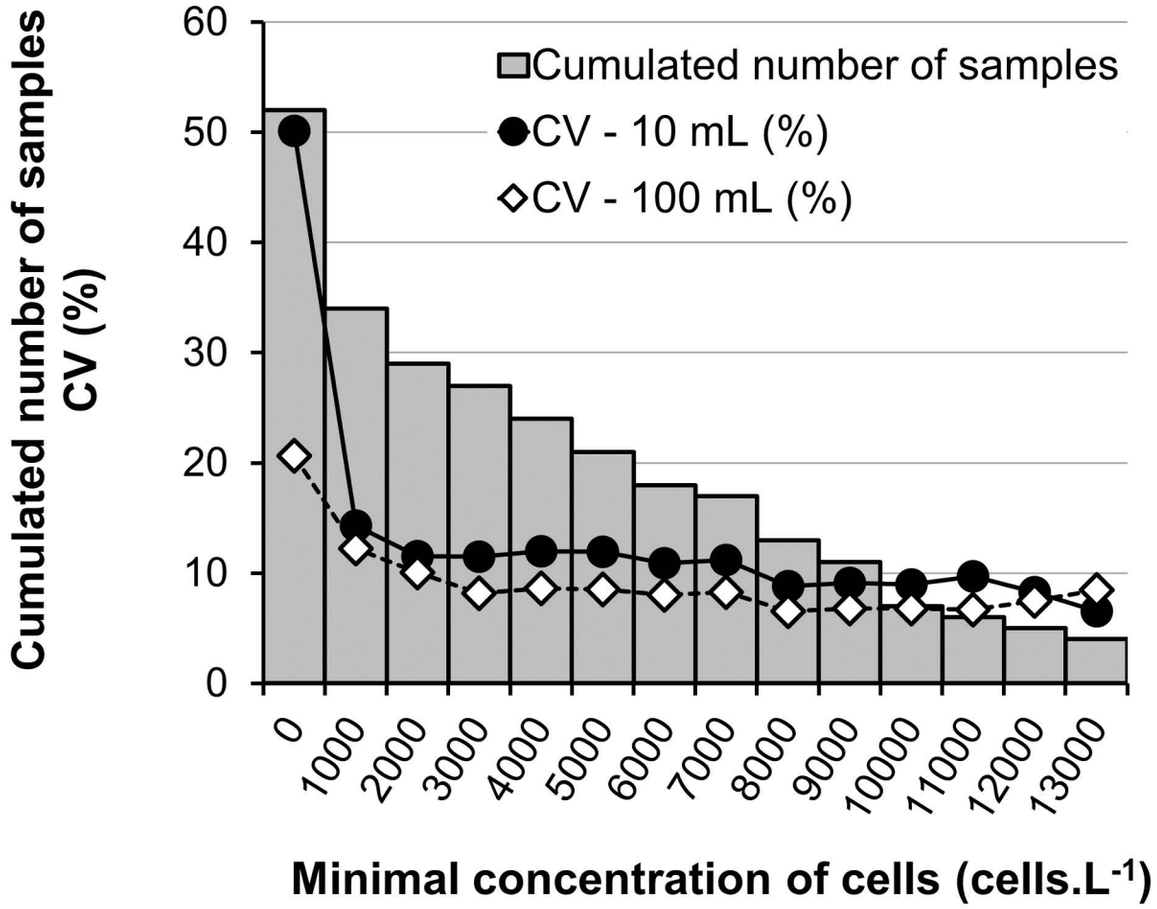
A


Concentration of cells compared to the 50 mL sedimentation sample (%)
400
300
200
100
0
10 mL column
0
3000
6000
9000
12000
15000
Concentration estimated with the 50 mL column (cells.L-1)

B


Concentration of cells compared to the 50 mL sedimentation sample (%)
250
200
150
100
50
0
100 mL column
0
3000
6000
9000
12000
15000
Concentration estimated with the 50 mL column (cells.L-1)

C


Cumulated number of samples CV (%)
60
50
40
30
20
10
0
Cumulated number of samples
CV - 10 mL (%)
CV - 100 mL (%)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
11000
12000
13000
Minimal concentration of cells (cells.L-1)

D

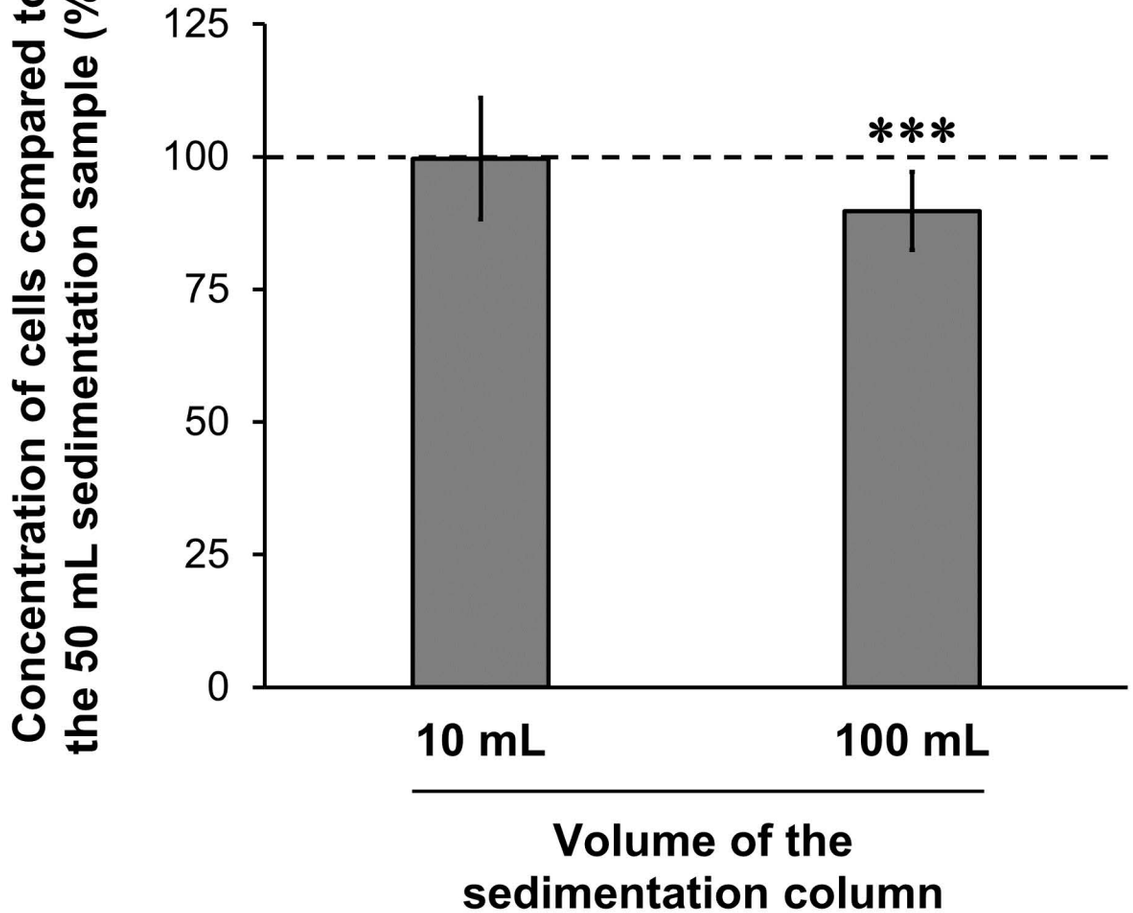

Concentration of cells compared to the 50 mL sedimentation sample (%)
125
100
75
50
25
0
***
10 mL
100 mL
Volume of the sedimentation column

Table 1

Site (country)	Coordinates		Typology of the site	*Ostreopsis* bloom characteristics					
	Latitude N	Longitude E		Timing				Maximal abundance of benthic cells	Macroalgal communities (main genera)
				Year	Start	Peak	End		
Rochambeau, Villefranche-sur-mer (France)	43°41'35"	7°18'31"	Natural rocky shore and protective stone boulders, sheltered environment	2014	06/25	07/09	08/06	529×10^3 cells.g^{-1} fw	Erect macroalgae *(Dictyota* spp., *Halopteris scoparia*)
				2015	06/25	07/16	07/23	813×10^3 cells.g^{-1} fw	Erect macroalgae (*Dictyota* spp., *Halopteris scoparia*)
Lanterne Beach, Nice (France)	43°40'38"	7°13'51"	Protective stone boulders along airport facilities	2015	07/2	07/16	08/27	740×10^3 cells.g^{-1} fw	Erect macroalgae (*Halopteris scoparia*, *Dictyota* spp.)
Passetto, Ancona (Italy)	43°36'35"	13°32'27"	Rocky coast and sheltered environment	2015	07/30	09/11	11/09	260×10^3 cells.g^{-1} fw	Erect macroalgae (*Halopteris scoparia*, *Dictyota* spp.)
Quarto, Genova (Italy)	44°23'17"	8°59'38"	Natural rocky shore, shallow, rather sheltered from sea wave	2015	06/25	07/04	08/12	2289×10^3 cells.g^{-1} fw	Erect macroalgae (*Halopteris scoparia*)
Salammbô (Tunisia)	36°50'35"	10°19'37"	Natural rocky shore, sheltered environment	2015	07/28	10/27	11/03	380×10^3 cells.g^{-1} fw	Erect macroalgae (*Sargassum* spp., articulated corallinales such as *Corallina* spp. and *Ellisolandia* spp.)

Batroun (Lebanon)	34°15'05"	35°39'25"	Natural rocky shore, rather sheltered from sea wave	2015	06/04	08/17	08/28	15×10^3 cells.g^{-1} fw	Erect macroalgae (articulated corallinales such as *Corallina* spp. and *Ellisolandia* spp.)

Table 2

	Level of abundance		Year of survey		Type of fixative condition	
	Significance	p value	Significance	p value	Significance	p value
Before agitation of the macroalgal sample	Yes	$p < 0.001$	Yes	$p < 0.001$	No	$p = 0.458$
After 10 sec of agitation of the macroalgal sample	No	$p = 0.052$	No	$p = 0.098$	Yes	$p < 0.001$
End of the 1st washing of the macroalgal substrate	No	$p = 0.065$	No	$p = 0.363$	Yes	$p < 0.001$
End of the 2nd washing of the macroalgal substrate	No	$p = 0.130$	No	$p = 0.805$	Yes	$p < 0.001$