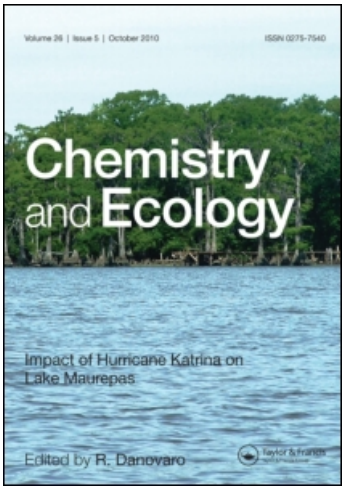

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis* (Poiret, 1789)

L. Valisano[a]; G. Bavestrello[b]; M. Giovine[c]; A. Arillo[a]; C. Cerrano[a]
[a] Dipartimento per lo Studio del Territorio e delle sue Risorse, Università di Genova, Genoa, Italy [b] Dipartimento di Scienze del Mare, Università Politecnica delle Marche, Ancona, Italy [c] Dipartimento di Biologia, Università di Genova, Genoa, Italy

To cite this Article Valisano, L. , Bavestrello, G. , Giovine, M. , Arillo, A. and Cerrano, C.(2007) 'Effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis* (Poiret, 1789)', Chemistry and Ecology, 23: 3, 233 — 241
To link to this Article: DOI: 10.1080/02757540701379634
URL: http://dx.doi.org/10.1080/02757540701379634

Chemistry and Ecology
Vol. 23, No. 3, June 2007, 233–241

Taylor & Francis
Taylor & Francis Group

Effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis* (Poiret, 1789)

L. VALISANO†, G. BAVESTRELLO‡, M. GIOVINE§, A. ARILLO† and C. CERRANO*†

†Dipartimento per lo Studio del Territorio e delle sue Risorse, Università di Genova, Corso Europa 26, 16132 Genoa, Italy
‡Dipartimento di Scienze del Mare, Università Politecnica delle Marche, Via Brecce Bianche, 60131, Ancona, Italy
§Dipartimento di Biologia, Università di Genova, Viale Benedetto XV, 16132 Genoa, Italy

(Received 29 November 2006; in final form 20 March 2007)

The suitability of the primmorphs system as a good model for biotechnological applications led researchers on primmorphs to look for a medium to stimulate cell proliferation and therefore growth of aggregates. Recent efforts have focused on the use of Fe^{+3} and Si that, supplemented to cell culture medium, were found to be promising for growth and morphogenesis of the sponge *Suberites domuncula*. In this work, we analysed the effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis*, by testing them at different concentrations in successive experiments. The purpose of these experiments was to test their effect on primmorphs and individuate their optimal concentration for this species. Our results suggest a negative effect of iron on primmorphs of *P. ficiformis* and a positive effect of silica on primmorphs size and spiculogenesis at a concentration of 120 μM.

Keywords: Cell culture; Mediterranean sponge; Primmorphs; Iron; Silica

1. Introduction

The culture of primmorphs is considered one of the most promising approaches for biotechnological applications of sponges. These round aggregates are formed with the re-establishment of cell–cell and cell–matrix contacts after sponge-cell dissociation and constitute the end of the aggregation of cellular material in primary lineages [1]. Contrary to dissociated cells, primmorphs are suggested to be characterized by a potential telomerasic activity [2, 3]. Despite this, primmorphs do not show effective cell proliferation, and growth rates are low [4]. The increase in size of primmorphs is generally due not to the production of new biomass, but owing to the fusion of small primmorphs into large new ones [5]. Cell culture *in vitro* therefore needs supplementary stimuli to initiate mitosis, and several authors have evidenced the lack of an appropriate growth medium [6] and growth promoters to produce new biomass in such lineages. A fetal bovine serum has been used in first attempts [7, 8], but serum easily causes

*Corresponding author. Email: cerrano@dipteris.unige.it

Chemistry and Ecology
ISSN 0275-7540 print/ISSN 1029-0370 online © 2007 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/02757540701379634

contaminant proliferation [9], which required the use of sterility and/or antibiotics. However, the use of antibiotics negatively affects the symbiotic equilibrium with heterotrophic bacteria, so that the use of these substances is preferentially avoided [4, 10, 11]. A variety of commercial media supplemented with sponge extracts, *Escherichia coli*, and bacteria isolated from sponges, soluble glucose, or lipids have been tested [12, 13]. Recent researchers have suggested that the addition of Fe^{3+} and Si to sea-water-based media induces not only a significant proliferation of cells in primmorphs, but also the expression of a cluster of genes, whose activation is correlated to the formation of spicules [14, 15]. This phenomenon is particularly important because the production of spicules can be considered as an early step in the morphogenesis and development of primmorphs into complete sponges [16–18].

Sponge spicules are rich in iron: a spicule of *Suberites domuncula* contains 1–7% iron (as Fe_2O_3) [18], while the extracellular concentration of Si controls the expression of the silicatein gene, directly related to the synthesis of spicules in both *S. domuncula* [18] and *Tethya aurantium* [19]. Sea water contains 1 nM of total Fe, while dissolved silica is present in sea water at a concentration of roughly 1–10 μM [18, 20, 21]. Whereas surface waters are relatively poor of dissolved silica, with a concentration always lower than 3 μM [21], deep sea waters are rich in $Si(OH)_4$, with varying concentrations, according to different authors, from 3 to 150 μM [22] or 10 to 180 μM [21].

Petrosia ficiformis was found, among Mediterranean sponges, to be one of the most productive species for primmorph formation [5], and so, as in previous reports [10], this species was chosen for improvement studies in primmorph production.

In this work, the effect of supplemented iron and dissolved silica, which led to a morphogenetic potential on primmorphs of *Suberites domuncula* [14, 18], was tested on primmorphs of *Petrosia ficiformis*, with the purpose to optimize culture conditions and induce an increase in primmorph size.

2. Materials and methods

2.1 *Specimen collection*

Specimens of *Petrosia ficiformis* were collected along the Marine Protected Area of Portofino (Ligurian Sea, Italy) at depths of 15–20 m. Sponges were immediately carried to the laboratory and maintained in aquaria at 12 °C and at a salinity of 38‰.

2.2 *Cell dissociation*

The day after sampling, sponges were processed for dissociation of cells according to the previous established protocol [3]. Sponge samples of 4–5 cm^3, continuously submersed in sea water, were cut into small pieces and transferred into 50-ml conical tubes filled with calcium- and magnesium-free sea water supplemented with EDTA (CMFSW-EDTA). After gentle shaking for 20 min, the solution was discarded, and new CMFSW-EDTA was added. After continuous shaking for 40 min, the supernatant was collected, filtered through a 40-μm-mesh nylon net, and the process repeated. Samples were centrifuged (1600 rpm, 458 g for 5 min) and washed twice in CMFSW. Cells in final pellets were resuspended in natural filtered sea water, and dissociated cells were put into tissue-culture plastic plates on an oscillating table to avoid cells attaching to the bottom of plates.

After 3 d, one-third of the sea water was replaced with new filtered water.

2.3 *Experimental conditions and data analysis*

According to previous reports [3], 5-d-old primmorphs were picked up, their radius measured under a stereomicroscope, and then put into different culture conditions to test the effect of supplemented iron (as ferric citrate) and dissolved silica (as Na-silicate) on their size.

In a first set of experiments, 5-d-old primmorphs were incubated in: (1) natural sea-water medium containing 5 μM silica as a control; (2) natural sea water supplemented with 30 μM Fe^{3+}; (3) natural sea water supplemented with 60 μM silica; and (4) natural sea water supplemented with 30 μM Fe^{3+} together with 60 μM silica.

In the second experimental set, the concentrations tested were increased to 60 μM Fe^{3+} and 120 μM silica. Therefore, in the second set of experiments 5-d-old primmorphs were incubated in: (1) natural sea-water medium containing 5 μM silica; (2) natural sea water supplemented with 60 μM Fe^{3+}; (3) natural sea water supplemented with 120 μM silica; and (4) natural sea water supplemented with 60 μM Fe^{3+} together with 120 μM silica.

As consequence of results obtained from the second experimental set, in the third set, we improved culture conditions by increasing the concentration of supplemented dissolved silica, but maintaining the concentration of supplemented iron constant.

In the third set of experiments 5-d-old primmorphs were incubated in: (1) natural sea-water medium containing 5 μM silica; (2) natural sea water supplemented with 180 μM silica; and (3) natural sea water supplemented with 30 μM Fe^{3+} together with 120 μM silica.

In the fourth set of experiments, all culture conditions were finally tested in parallel. 5-d-old primmorphs were incubated in: (1) natural sea-water medium containing 30 μM Fe^{3+} and 60 μM silica; (2) natural sea water supplemented with 30 μM Fe^{3+} and 120 μM silica; (3) natural sea water supplemented with 30 μM $Fe^{3+}$and 180 μM silica; (4) natural sea water supplemented with 120 μM silica; and (5) natural sea water supplemented with 180 μM silica.

We did six replicates for all experimental conditions of each experimental set. The concentration of dissolved silica of natural sea water of the experimental sets was assessed using a silica (silicic acid) test (Merck).

Every 3 d, one-third of the sea water was replaced with new filtered water, always supplemented with iron or dissolved silica, according to each experimental set. At each water change, primmorphs were measured under a stereomicroscope, and the effect of supplemented ions on the size of the primmorphs was monitored for a month.

Areas of primmorphs were calculated measuring their diameters and assuming primmorphs to be perfect spheres [4]. In each experimental set, the effect of treatments was tested using the Jonckheere test, Kruskall–Wallis ANOVA, and, when appropriate, a multicomparison analysis [23].

At the end of the final experiments, after a month of supplementing ions, primmorphs were picked up for spicule analysis using an optical microscope. Primmorphs of the same size (1–1.5 mm) and from separated samples for each culture condition were placed onto glass slides and observed at the optic microscope. Spicules were identified in the primmorphs thanks to their bright light reflection properties [15]. The length and thickness of the spicules within primmorphs were recorded and compared using Mood's Median Test.

3. Results

The concentration of Si and Fe^{3+} (60 μM and 30 μM, respectively) tested in the first experimental set (figure 1A) induced a negligible increase in size in primmorphs of *Petrosia ficiformis*. From a comparison of size of primmorphs under the different conditions, we found a very

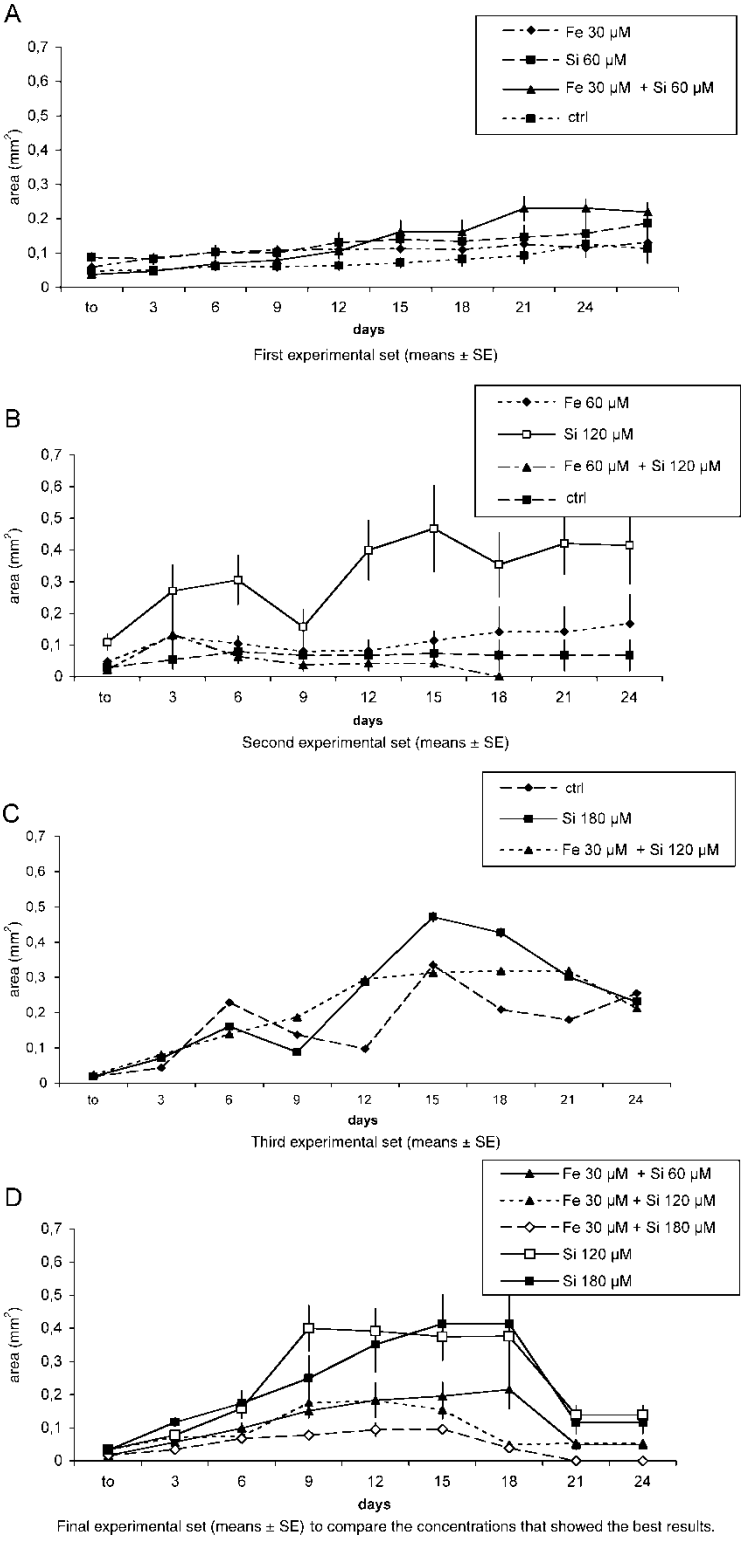

Figure 1. Maintenance of primmorphs with different salt concentrations.

slight treatment effect (Kruskall–Wallis ANOVA: $P \leq 0.05$), with no differences between effects of supplementing of the iron alone and of the silica alone.

In the second experimental set (figure 1B), the increase in concentration of both ions resulted to induce a more evident effect (Kruskal–Wallis ANOVA: $0.02 < P < 0.05$). With 120 μM Si primmorphs reached the highest dimension of $0.46 \pm 0.13\ mm^2$ (multicomparison analysis: $P < 0.05$). The supplementation with only 60 μM iron does not show any variation in comparison with the control. Iron added with silica caused disintegration of primmorphs in a couple of weeks. For successive experiments, these results suggest that a lower concentration of iron (30 μM) should be used.

The further increase in dissolved silica in sea water medium (figure 1C) showed a more significant effect (Kruskall–Wallis ANOVA: $0.001 < P < 0.014$). Increasing the concentration of Si to 180 μM, primmorphs reached a size of $0.47 \pm 0.12\ mm^2$. In this experimental set, 180 μM Si induced the greatest size increase in the primmorphs (multicomparison analysis: $P < 0.05$).

The final test (figure 1D) allowed us to show a significant increase in primmorph size in relation to the increase in silica concentration (Jonckheere test: $P < 0.05$) and a highly significant treatment effect (Kruskall–Wallis ANOVA: $0.001 < P < 0.01$). The optimal conditions for cultures of primmorphs were obtained with Si at concentrations of 120 μM and 180 μM, while the further adding of Fe^{3+} negatively affect the size of primmorphs (multicomparison analysis $P < 0.05$). After 18 d of culture at the concentrations of 120 μM and 180 μM Si, we observed an increase in size of $0.37 \pm 0.07\ mm^2$ and $0.41 \pm 0.08\ mm^2$, respectively, with no differences between treatments. The size reached was not maintained by primmorphs for a long time, but even if sea water was renewed, cultures were made up by keeping the concentration of ions in each experimental set stable, and the size of the primmorphs after 21 d dramatically decreased. Therefore, the results obtained allowed us to demonstrate a null or negative effect of the combinations of the two ions on cell cultures and a short time effect at Si concentrations of 120 μM and 180 μM.

Ions concentration was found to have an effect not only on the size of primmorphs, but also on spicule production within primmorphs. The different concentrations tested in the final experimental set (figure 2) showed a very significant effect on the length of spicules formed

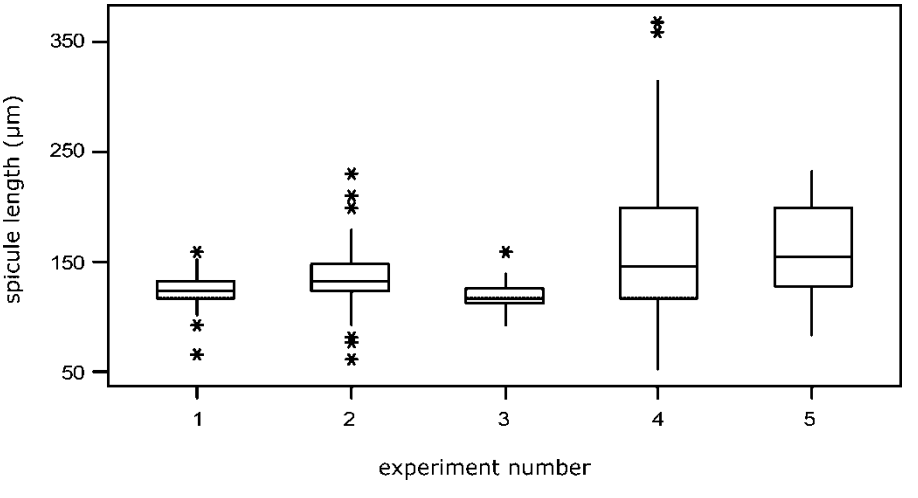

Figure 2. Spicules length (medians ± IC) in relation to ions concentrations tested (1: Fe 30 μM + Si 60 μM; 2: Fe 30 μM + Si 120 μM; 3: Fe 30 μM + Si 180 μM; 4: Si 120 μM; 5: Si 180 μM). High-resolution outputs mark the distance of values from IC range with asterisks.

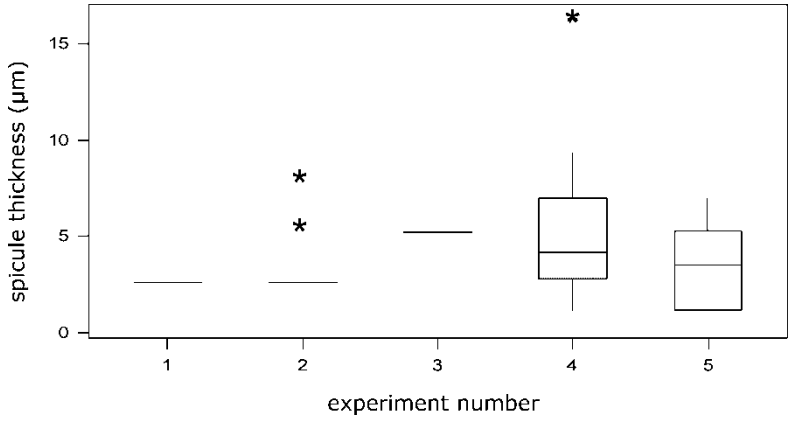

Figure 3. Spicules thickness (medians ± IC) in relation to ions concentrations tested (1: Fe 30 µM + Si 60 µM; 2: Fe 30 µM + Si 120 µM; 3: Fe 30 µM + Si 180 µM; 4: Si 120 µM; 5: Si 180 µM).

(Mood's median test: $P = 0.000$). In the case of Si combined with Fe^{3+}, spicules were shorter in length than those shown with only the silica. With Fe^{3+} combined with 120 µM and 180 µM Si, spicule lengths were on average 128.09 µm and 119.0 µm, respectively. With Fe^{3+} and 120 µM Si, the spicule lengths were higher but always lower than 134.7 µm (mean value). With the only silica spicules joined the length of 155.8 µm (mean value) and 159.6 µm (mean value), with 120 µM and 180 µM Si, respectively. In particular we demonstrated, as shown in figure 4, that the spicule lengths were greater and more variable with 120 µM and 180 µM Si compared with cultures with iron, thus showing that the length of the spicules formed within primmorphs is negatively affected by the presence of iron in cell cultures and varies depending on the availability of dissolved silica. The thickness of the spicules produced (figure 3) was also higher in the presence of 120 µM and 180 µM Si compared with the conditions with the two ions combined (Mood's median test: $P = 0.002$).

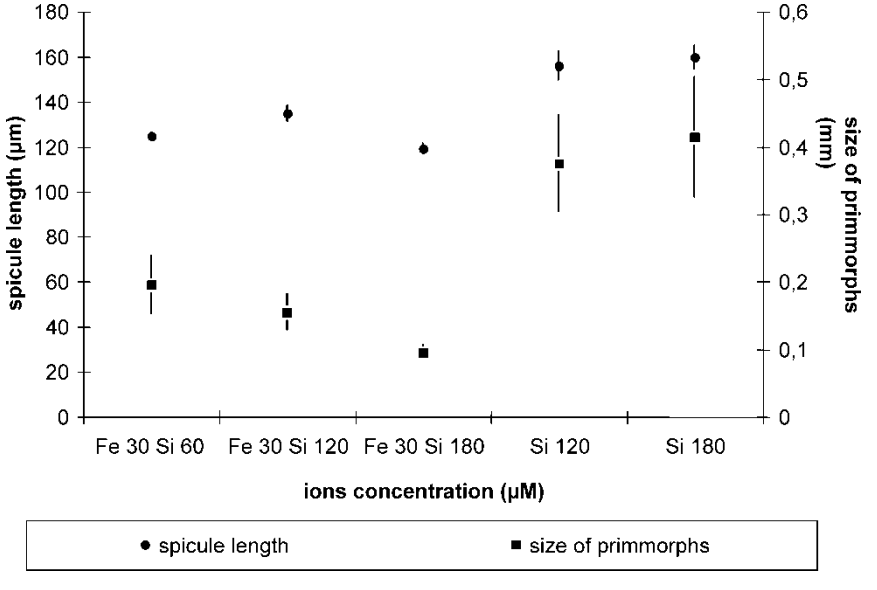

Figure 4. Trends of spicules length and size of primmorphs in relation to ions concentration tested (means ± SE).

The results obtained allowed us to show the same effect of ion concentrations on the two parameters studied on primmorphs (figure 4). In both cases, the presence of iron negatively affected cultures. The increase in dissolved silica in the presence of iron was found not to promote an increase in the size of primmorphs and the length of the spicules produced.

Therefore, the combination of the two ions Fe^{3+} and Si was found to have a negative affect on cell cultures for both parameters analysed, while the availability of the only dissolved silica produced a response in both cases. Moreover, in culture conditions with 120 μM Si, the high variability of spicule size formed by primmorphs is coupled with a more variable range of spicular types. While under the other conditions, we found only hastate, blunt, or curved oxeas, in cultures with 120 μM Si, we found curved and stepped oxeas, strongyles, and even styles within primmorphs.

4. Discussion

The addition of iron and silica in sea-water-based culture medium affected the growth dynamics of primmorphs of *Petrosia ficiformis*. Unlike *S. domuncula*, which, in the presence of 60 μM Si, tripples the size of primmorphs from 2 to 6 mm [14], and, with 30 μM Fe and 60 μM Si, increases the size to 10 mm [24], in *P. ficiformis* the concentration of 30 μM Fe and 60 μM Si stimulates a slight increase in size.

The series of successive experiments that we performed allowed us to determine the optimal culture conditions to induce an increase in primmorph size for this species, albeit not comparable with the increase in size reported for *S. domuncula*.

Our results showed that supplementing sea-water medium with the combination of iron and silica the primmorphs of *P. ficiformis* growth is limited or completely compromised.

The null effect of iron on the size of the primmorphs has already been demonstrated for the species *Hymendiacidon perleve* [25]. Therefore, as previously suggested [25], the effect of iron on cell cultures may be species-dependent. Good results have been obtained by increasing the concentration of the dissolved silica only.

With silica at a concentration of 120 μM, primmorphs showed an increase in size. A further increase in the concentration of available dissolved silica resulted in a null effect on the size of primmorphs. These results were confirmed in the analyses of spicules produced within primmorphs.

To date, the effect of silica on spicule formation *in vitro* has been determined only by a semi-quantitative approach, using the same concentrations of iron and silica tested for primmorphs size, showing that iron influences spicule production in *S. domuncula* [24].

The availability of dissolved silica has a great influence on the formation of spicules for the sponge *P. ficiformis*, but also in this case, the presence of iron had a negative influence on the length of spicules. The length and thickness of spicules formed under the different conditions suggested that the presence of iron in sea-water-based medium cultures could inhibit the deposition of silica in *P. ficiformis* primmorphs.

Therefore, whereas, for *S. domuncula* primmorphs, iron induces cell proliferation and morphogenetic effects with an accelerative and supportive influence on spicule synthesis [14], for *P. ficiformis* iron seems to be unnecessary and to have a negative effect on the growth and morphogenesis of primmorphs, particularly when added with silica.

This may be related to the iron concentration the sponge experiences in its natural environment: if the iron concentration in the area of sponge sampling is low, the iron supplementation in cultures at concentrations higher than that usually present in nature could lead to an overexpression of genes involved in spiculogenesis, and consequently lead to metabolic stress.

The concentration of dissolved silica at 60 μM is the optimal concentration for the production of spicules in *S. domuncula* [26]. For the species *P. ficiformis*, the optimal concentration was found to be 120 μM. The spicule length was higher in the presence of silica only, with no great differences between 120 μM and 180 μM Si.

Our results suggest that in the case of *P. ficiformis*, the sizes of both primmorphs and spicules formed within primmorphs depend on the presence of silica. The concentration of dissolved silica influences silicatein gene expression; after exposure to silicic acid, a gene battery correlated to silicatein became expressed in concert, and spicules were formed [20]. The optimal silica concentration for synthesis of spicules is between 5 and 100 μM [21]. From results obtained to date, great differences can be found among species, suggesting that the concentration of available silica influencing primmorph size and spiculogenesis could be species-dependent.

The optimal concentration of 120 μM Si demonstrated was found to lead to not only a wider dimensional range of spicules but also a greater variation of spicular types in primmorphs of *P. ficiformis*. The variability of spicular-type production depending on the availability of dissolved silica could highlight several implications concerning the use of spicule type and morphology as taxonomic characters. Concerning this demonstrated aspect, the different production of spicular types depending on the availability of dissolved silica, as first observed in *C. crambe* [21], was never observed in the primmorph system. The lack of a response with the increase in availability of dissolved silica from 120 μM to 180 μM is in agreement with previous reports [21], in which the authors suggested that sponges' take-up of silica is more efficient in medium-Si conditions than in high-Si conditions and that it may be inhibited at unusually high concentrations [21, 27]. Transport of silica from extracellular spaces into cells occurs in co-transport with Na [28]. Sponges in effect have an efficient system to take up silica from the environment [20, 29], and high silica concentrations may inhibit the function of the mechanism of Na–Si exchange [30].

References

[1] M.R. Custodio, I. Prokic, R. Steffen, C. Koziol, R. Borojevic, F. Brümmer, M. Nickel, W.E.G. Müller. Primmorphs generated from dissociated cells from the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death. *Mech. Ageing. Dev.*, **105**, 45–59 (1998).

[2] C. Koziol, R. Borojevic, R. Steffen, W.E.G. Müller. Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. *Mech. Ageing. Dev.*, **100**, 107–120 (1998).

[3] W.E.G. Müller, M. Wiens, R. Batel, R. Steffen, H.C. Schröeder, R. Borojevic, M.R. Custodio. Establishment of a primary cell culture from a sponge: primmorphs from *Suberites domuncula*. *Mar. Ecol. Prog. Ser.*, **178**, 205–219 (1999).

[4] D. Sipkema, R. Van Wielink, A.A.M. Van Lammeren, J. Tramper, R. Osinga, R.H. Wijffels. Primmorphs from seven marine sponges: formation and structure. *J. Biotechnol.*, **100**, 127–139 (2003).

[5] L. Valisano, G. Bavestrello, M. Giovine, C. Cerrano. Primmorphs formation dynamics: a screening among Mediterranean sponges. *Mar. Biol.*, DOI: 10.1007/s00227-006-0297-1 (2006).

[6] M. Nickel, F. Brümmer. in vitro sponge fragment culture of *Chondrosia reniformis* (Nardo, 1847). *J. Biotechnol.*, **100**, 147–159 (2003).

[7] S.A. Pomponi, R. Willoughby. Sponge cell culture for production of bioactive metabolites. In *Sponges in Time and Space*, R. van Soest, T.M.G. van Kempen, J.C. Brakman (Eds), pp. 395–400, Balkema, Rotterdam, Netherlands (1994).

[8] M. Ilan, H. Contini, S. Carmeli, B. Rinkevich. Progress towards cell cultures from a marine sponge that produces bioactive compounds. *J. Mar. Biotechnol.*, **4**, 145–149 (1996).

[9] R. Osinga, J. Tramper, R.H. Wijffels. Cultivation of marine sponges. *Mar. Biotechnol.*, **1**, 509–532 (1999).

[10] L. Valisano, G. Bavestrello, M. Giovine, A. Arillo, C. Cerrano. Seasonal production of primmorphs from the marine sponge *Petrosia ficiformis* (Poiret, 1789) and new culturing approaches. *J. Exp. Mar. Biol. Ecol*., DOI: 10.1016/j.jembe.2006.06.030 (2006).

[11] M. Pozzolini, L. Sturla, C. Cerrano, G. Bavestrello, L. Camardella, A.M. Parodi, F. Raheli, U. Benatti, W.E.G. Müller, M. Giovine. Molecular cloning of silicatein gene from the sponge *Petrosia ficiformis* (Porifera,

Demospongia) and development of primmorphs as a model for biosilification. *Mar. Biotechnol.*, **6**, 594–603 (2004).
[12] M. Klatau, M.R. Custodio, R. Borojevic. In vitro culture of primary cell lines from marine sponges. In *Sponges in Time and Space*, R. van Soest, T.M.G. van Kempen, J.C. Brakman (Eds), *Sponges in Time and Space*, pp. 401–406, AA Balkema, Rotterdam. The Netherlands (1994).
[13] S. De Rosa, S. De Caro, C. Iodice, G. Tommonaro, K. Stefanov, S. Popov. Development in primary cell culture of Demosponges. *J. Biotechnol.*, **100**, 119–125 (2003).
[14] G. Le Pennec, S. Perovic, M.S.A. Ammar, V.A. Grebenjuk, R. Steffen, F. Brummer, W.E.G. Müller. Cultivation of primmorphs from the marine sponge *Suberites doumuncula:* morphogenetic potential of silicon and iron. a review. *J. Biotechnol.*, **100**, 93–108 (2003).
[15] H.C. Schröeder, S. Perovic-Ostadt, V.A. Grebenjuk, S. Engel, I.M. Müller, W.E.G. Müller. Biosilica formation in spicules of the sponge *Suberites domuncula*: synchronous expression of a gene cluster. *Genomics,* **85**, 666–678 (2005).
[16] W.E.G. Müller, M. Bohm, R. Batel, S. De Rosa, G. Tommonaro, I.M. Müller, H.C. Schröeder. Application of cell culture for the production of bioactive compounds from sponges: synthesis of Avarol from *Dysidea avara. J. Nat. Prod*., **63**, 1077–1081 (2000).
[17] H.C. Schröeder, A. Krasko, R. Batel, A. Skorokhod, S. Pahler, M. Kruse, I.M. Müller, W.E.G. Muller. Stimulation of protein (collagen) synthesis in sponge cells by a cardiac myotropin-related molecule from *Suberites domuncula*. *FASEB J*., **14**, 2022–2031 (2000).
[18] A. Krasko, H.C. Schröeder, R. Batel, V.A. Grebenjuk, R. Steffen, W.E.G. Müller. Iron induces proliferation and morphogenesis in primmorphs from the marine sponge *Suberites domunucula*. *DNA Cell. Biol.*, **21**, 67–80 (2002).
[19] K. Shimizu, J. Cha, G.D. Stucky, D.E. Morse. Silicatein alpha: cathepsine L-like protein in sponge biosilica. *Proc. Natl. Acad. Sci. USA,* **95**, 6234–6238 (1998).
[20] H.C. Schröeder, S. Perovic-Ostadt, M. Rothenberger, M. Wiens, H. Schwertner, R. Batel, M. Korzhev, I.M. Müller, W.E.G. Müller. Silica transport in the Demosponge *Suberites domuncula*: fluorescence emission analysis using the PDMPO probe and cloning of a potential transporter. *Biochem. J.*, 381, 665–673 (2004).
[21] M. Maldonado, M.C. Carmona, M.J. Uriz, A. Cruzado. Decline in Mesozoic reef building sponges explained by silicon limitation. *Nature*, **401**, 785–788 (1999).
[22] Kennish. *Practical Hand-Book of Marine Science*, CRC Press, Boca Raton, FL (1994).
[23] S. Siegel, N.J. Castellan. *Statistica Non Parametrica*, Caracciolo Editore Caracciolo Editore, Mc Graw-Libri, Milano, Italia (1988).
[24] A. Krasko, R. Batel, H.C. Schröeder, I.M. Müller, W.E.G. Müller. Expression of silicatein collagen genes in the marine sponge *Suberites domuncula* is controlled by silicate and myotrophin. *Eur. J. Biochem*., **167**, 4878–4887 (2000).
[25] Q. Zhao, W. Zhang, M. Jin, X. Yu, M. Deng. Formulation of a basal medium for primary cell culture of the marine sponge *Hymeniacidon perleve*. *Biotechnol. Prog*., **21**, 1008–1012 (2005).
[26] T.L. Simpson. *The Cell Biology of Sponges*, Springer, New York (1984).
[27] M.J. Uriz. Mineral skeletogenesis in sponges. *Can. J. Zool*., **84**, 322–356 (2006)
[28] M. Hildebrand, B.E. Volcani, W. Gassmann, J.I. Schröeder. A gene family of silicon transporters. *Nature,* **385**, 688–689 (1997).
[29] W.E.G. Müller, S.I. Belikov, W. Tremel, C. Perry, W.W.C. Gieskes, A. Boreiko, H.C. Schröder. Siliceous spicules in marine Demosponges (example *Suberites domuncula*). *Micron*, **37**, 107–120 (2006).
[30] T. Coradin, P.J. Lopez. Biogenic silica patterning: simple chemistry or subtle biology? *ChemBioChem.*, **4**, 251–259 (2003).