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COOPERATION

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1. COCCOLITHOPHORES:

1.1 Intracellular pH homeostasis (Partner 11: MBA)
This work is closely linked with D 4.2 *"Identify the molecular components of the main DIC uptake mechanisms in coccolithophores with a combination of in silico and functional approaches"* that was reported in M36.

Single cell studies of intracellular pH regulation and calcification have provided substantial novel data on the physiological regulation of intracellular pH in *Coccolithus pelagicus*. *In vivo* imaging of intracellular pH, using confocal microscopy and fluorescent pH indicators, has been carried out in relation to the role of a novel pH regulatory mechanism that involves a newly-discovered class of plasma membrane ion channels (H^+ channels) in photosynthetic eukaryotes. This work, published in *PLoS Biology*, has shown that *C. pelagicus* does not maintain a constant intracellular pH in the face of short-term fluctuations in external pH. Our findings parallel those from similar experiments from Ribebsell's group at IFM Geomar, Kiel [PARTNER] (Suffrian et al, 2011, *New Phytologist* 190, 595-608) with the coccolithophore *Emiliania huxleyi*. The conclusions are consistent with recent evolution of coccolithophores in an external pH environment that has shown little significant major pH fluctuation and the absence of selection of cellular pH regulatory mechanisms in response to eternal pH changes. In contrast, we have shown from simultaneous patch clamp electrophysiology and pH imaging experiments that intracellular pH can be regulated very effectively by changes in membrane potential and intracellular pH. This is consistent with the operation of a voltage-regulated, H+ channel that senses changes in intracellular pH as a major short-term regulatory mechanism for cellular pH. We have identified and cloned the H^+ channel gene, expressed it in a heterologous system (human HEK cells) and shown that is it highly selective for H^+. These findings present a new and unexpected aspect of coccolithophore physiology. Our phylogentic analyses of H^+ channel genes shows that they are present in other phytoplankton groups and probably pay a key role in intracellular pH regulation more generally in phytoplankton. We are beginning to consider the implications of ocean acidification in the functioning of H^+ channels more generally.

In order to assess the importance of pH regulation in the calcification process in coccolithophores we developed a novel, simple and extremely sensitive method of quantifying intracellular calcite production in single coccolithophore cells or groups of cells *in vivo*. This method relies on the birefengence properties of calcite and uses quantitative polarised light microscopy to monitor the amount of calcite production. The method shows that coccolithophores produce calcite at linear rates under constant light conditions. However, this rate is significantly reduced by very brief treatments that perturb intracellular pH homeostasis. For example, as little as 5 minutes treatment with a weak acid that causes a reversible reduction in intracellular pH produces a prolonged (up to 2 hours) inhibition of calcite production. There are two major implications of these findings: First, calcification has an absolute requirement for regulated cytoplasmic pH. Second, higher level regulatory mechanisms come into play to shut down the calcification machinery in the face of cytosolic acidosis and this inhibition of calcification remains for significantly longer than the acidosis. The underlying mechanisms of this important regulatory process will be the subject of future studies.

A further finding from this work is that *E. huxleyi* cells cultured for prolonged periods at lower pH (7.8) in pH-stats lose the passive response of intracellular pH to imposed fluctuations in external pH. This implies that the H+ permeability of coccolithophores grown at lower pH values is reduced as part of the physiological acclimation mechanism. The molecular mechanisms underlying this regulation are currently being investigated

The findings described above have led to the recognition of the importance of understanding pH in the diffusion-limited layer of the cell surface in interpretations of the effects of changes in external pH on the H^+ gradients across the cell membrane and the carbon speciation at the cell surface. A collaborative modelling and experimental programme has been initiated to test a number of hypotheses relating to the regulation of intracellular and cell surface pH in relation to metabolic processes in phytoplankton more generally.

1.2 Membrane permeability (Partner 3: IFM_Geomar).
To understand the influence of changing surface ocean pH and carbonate chemistry on the coccolithophore *Emiliania huxleyi*, it is necessary to characterize mechanisms involved in pH homeostasis and ion transport. Here, we measured effects of changes in seawater carbonate chemistry on the fluorescence emission ratio of BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carb- oxyfluorescein) as a measure of intracellular pH (pHi). Out of equilibrium solutions were used to differentiate between membrane permeation pathways for H^+, CO_2 and HCO_3). Changes in fluorescence ratio were calibrated in single cells, resulting in a ratio change of 0.78 per pHi unit. pHi acutely followed the pH of seawater (pHe) in a linear fashion between pHe values of 6.5 and 9 with a slope of 0.44 per pHe unit. pHi was nearly insensitive to changes in seawater CO2 at constant pHe and HCO3). An increase in extracellular HCO3) resulted in a slight intracellular acidifi- cation. In the presence of DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid), a broad-spectrum inhibitor of anion exchangers, E. huxleyi acidified irreversibly. DIDS slightly reduced the effect of pHe on pHi. The data for the first time show the occurrence of a proton permeation pathwayin *E. huxleyi* plasma membrane. pHi homeostasis involves a DIDS-sensitive mechanism.

1.3 Response of coccolithophores to elevated CO_2

1.3.1 Molecular responses to ocean acidification (partner 24: SOTON-SOES)
Shotgun proteomic analysis of the marine coccolithophore *Emiliania huxleyi* revealed the presence of multiple proteins that may have roles in the calcification process, including house keeping proteins such as clathrin that may be utilized in coccolithogenesis (Jones et al., 2010). This paper describes the methods for the extraction of proteins from *E. huxleyi* for proteomic studies, and an survey of its proteome. By fractionating proteins using SDS-PAGE and subsequently analyzing the corresponding tryptic peptides by nano-LC coupled to tandem MS (GeLC-MS/MS), protein were identified. A software pipeline "Bioinformatics Utility for Data Analysis of Proteomics using ESTs" (BUDAPEST) - that allows the robust identification of proteins from expressed sequence tags (ESTs) using MS/MS data was developed. While little information is available on the genome of *E. huxleyi*, there are currently sequence data from laboratory experiments for over 90,000 ESTs, which were used in this study. MS-based proteomic technologies

could allow large-scale studies on *E. huxleyi* at the protein level to characterize its global response to changes in environmental conditions.

Links with WP9 and WP11: Links with WP9 and WP11 are established to contribute to a database of past and current OA experiments. This is open access to EPOCA partners and it is continuously being updated (see current compilation of published data on the impact of OA on organisms and ecosystems and lead to a meta-analysis paper (Gehlen et al., in press; Oschlies et al., in press).

1.3.2 Impact of ocean acidification on calcification of coccolithophores *(Partner 1.1: LOV).* Inorganic carbon content and production related to pCO_2 environment (400 and 760 µatm) were investigated in the diploid life stage of three widespread coccolithophores (*E. huxleyi, C. leptoporus,* and *S. pulchra*). Elevated pCO_2 had no significant effect on both parameters in *C. leptoporus* and *S. pulchra* while it increased inorganic carbon content and production in the *E. huxleyi* strain. Physiological and ecological consequences of these results have been interpreted (Fiorini et al., 2010, in press)

1.3.3 Separating the effects of different carbonate system parameters *(Partner 3: IFM-Geomar)* To distinguish the effects of individual carbonate system parameters on growth, primary production, and calcification in coccolithophores, we cultured *Emiliania huxleyi* under a broad range of carbonate chemistry conditions. In a first experiment, we kept total alkalinity constant and varied seawater pCO_2 from 20 to 6000 µatm in between different treatments. This experiment was performed to investigate whether growth, calcification and photosynthesis respond linearly to increasing CO_2 and decreasing pH (as in ocean acidification) or if these processes have an optimum at defined carbonate chemistry conditions. In a second experiment, we increased pCO_2 as in the first one, but kept pH instead of total alkalinity constant (pH_{free}=8). The second experiment and the comparison with the first experiment enabled us to distinguish the individual influence of different components of the carbonate chemistry (CO_2, HCO_3^-, CO_3^{2-} and pH). Results of the constant alkalinity approach revealed physiological optima for growth, calcification and organic carbon production at pCO_2 values of ~200 µatm, ~400 µatm, and ~800 µatm, respectively. Comparing this with the constant pH approach showed that growth and organic carbon production increased similarly from low to intermediate CO_2 levels but started to diverge towards higher CO_2 levels. In the high CO_2 range, growth rates and organic carbon production decreased steadily with declining pH at constant alkalinity while remaining consistently higher at constant pH. This suggests that growth and organic carbon production rates are directly related to CO_2 at sub-saturating concentrations whereas towards higher CO_2 levels they are adversely affected by the associated decrease in pH. A pH dependence at high pCO_2 is also indicated for calcification rates, while the key carbonate system parameter determining calcification at low fCO_2 remains unclear. Our findings imply that key metabolic processes in coccolithophores have their optima at different carbonate chemistry conditions and are influenced by different parameters of the carbonate system.

1.3.4 Calcidiscus leptoporus morphology in response to carbonate chemistry changes *(Partner 5: AWI).* CO_2 perturbation experiments are the prime tools to mimic future CO_2 scenarios and to study organism responses. The most common perturbation methods, leading to very similar speciation with regard to pH, [CO_2], [CO_3^{2-}] and Ω, are the dissolved inorganic carbon (DIC) manipulation by aeration with gasses of a certain

pCO_2 (while keeping total alkalinity (TA) constant) and manipulation of the TA by the addition of HCl or NaOH (while DIC stays constant). Even though TA and DIC manipulations differ with regard to the changed quantity, they result in very similar carbonate speciation. As in practically all CO_2 perturbation studies one of these methods was used, it is not known which parameter of the carbonate system causes observed effects. The development of a process-understanding of acidification effects, however, requires this knowledge. To disentangle putative CO_2, CO_3^{2-} concentration, HCO_3^- concentration, pH, TA, and DIC effects, experiments with *Calcidiscus leptoporus* were conducted in which the carbonate chemistry was not only modified using the standard techniques, i.e. TA and DIC manipulation, but additionally TA and DIC were modified in concert. This allowed e.g. variable CO_2 at constant pH. A suitable combination of different manipulations allowed us to identify the parameter of the carbonate system causing coccolith malformations. This parameter is CO_2.

2. BIVALVES AND BENTHIC CALCIFIERS

2.1 The Greenland smoothcockle *Serripes groenlandicus* in a warming more acidic ocean (Partner 5: AWI).

High latitude calcifiers might be severely affected by climate change, since the Arctic Ocean experiences the strongest warming and acidification trends and might become undersaturated with respect to aragonite already by the year 2050. The Greenland smoothcockle *Serripes groenlandicus* is a very important food source for walruses and has a function in bioturbation and, therefore, an important role in the Arctic food web. We studied the synergistic impacts of ocean acidification and ocean warming on acid-base regulation and on shell growth and shell structure of this arctic bivalve from an Arctic fjord in Svalbard (79°N, 12°E). We investigated extracellular and intracellular pH regulation and shell growth under different acidification and warming scenarios. We examined the microstructure of the shell to reveal differences in the ability to build calcium carbonate composites when the seawater is already corrosive.

S. groenlandicus were cultured for nine weeks under up to four different seawater PCO_2 levels ranging from 380 to 3000 µatm and under three different temperatures, namely 1, 4 and 7°C. Water aragonite was undersatured in all CO_2 treatments (≥750µatm) besides controls (380 µatm). Extra- and intracellular acid-base status (in muscle) were studied under high CO_2 levels and increased temperature over a time period of nine weeks. Bivalve extracellular fluids are well known to be poorly buffered, displaying high PCO_2 and low pH and carbonate levels. Haemolymph samples of *S. groenlandicus* were analyzed for pH and total dissolved inorganic carbon to calculate PCO_2 and bicarbonate [HCO_3^-]. Determination of the non-bicarbonate buffer capacity resulted in a very low value of 0.6 ± 0.1 mmol/l/pH, in line with earlier findings in marine bivalves from temperate zones. No accumulation of extracellular bicarbonate was measured under elevated CO_2 and the pH disturbances followed the non-bicarbonate buffer line. Accordingly, changes in extracellular pH were similar to those of seawater pH. Extracellular PCO_2 was always above seawater PCO_2 and the difference between seawater and extracellular space consistently ranged around 0.04 to 0.05 kPa, driving CO_2 release by diffusion. In contrast to extracellular pH, intracellular acid-base status in the foot muscle did not show any CO_2 effect, but a slight decrement in pH with temperature following the alphastat pattern. The tissue buffering capacity was determined to be -15.5 ± 2.0 mmol/l/pH in the foot muscle of *S. groenlandicus,* also in line with earlier findings.

2.2 Impact of ocean acidification of the shell thickness of mussels (Partner 1.1: LOV)

Several experiments have shown a decrease of growth and calcification of organisms at decreased pH levels. There is a growing interest to focus on early life stages that are believed to be more sensitive to environmental disturbances such as hypercapnia. Here, we present experimental data, acquired in a commercial hatchery, demonstrating that the growth of planktonic mussel (Mytilus edulis) larvae is significantly affected by a decrease of pH to a level expected for the end of the century. Even though there was no significant effect of a 0.25–0.34 pH unit decrease on hatching and mortality rates during the first 2 days of development nor during the following 13-day period prior to settlement, final shells were respectively 4.5±1.3 and 6.0±2.3% smaller at pHNBS~7.8 (pCO2~1100–1200 µatm) than at a control pHNBS of ~8.1 (pCO2~460–640 µatm). Moreover, a decrease of 12.0±5.4% of shell thickness was observed after 15d of development. More severe impacts were found with a decrease of ~0.5 pHNBS unit during the first 2 days of development which could be attributed to a decrease of calcification due to a slight undersaturation of seawater with respect to aragonite. Indeed, important effects on both hatching and D-veliger shell growth were found. Hatching rates were 24±4% lower while D-veliger shells were 12.7±0.9% smaller at pHNBS~7.6 (pCO2~1900 µatm) than at a control pHNBS of ~8.1 (pCO2~540 µatm). Although these results show that blue mussel larvae are still able to develop a shell in seawater undersaturated with respect to aragonite, the observed decreases of hatching rates and shell growth could lead to a significant decrease of the settlement success. As the environmental conditions considered in this study do not necessarily reflect the natural conditions experienced by this species at the time of spawning, future studies will need to consider the whole larval cycle (from fertilization to settlement) under environmentally relevant conditions in order to investigate the potential ecological and economical losses of a decrease of this species fitness in the field. (Gazeau et al., 2010)

2.3 Responses of benthic calcifiers (Partner 1.1: LOV).

The organisms living in benthic environments and their ecosystems are highly variable as they include the full range of the oceans with associated changes in physical and chemical conditions driven by depths and latitudinal and geographic variations. Large-scale geographical differences in seawater carbonate chemistry of natural and anthropogenic origin was used by Andersson et al (in press) to assess the effect of ocean acidification on marine benthic systems. In benthic environments, the three typically occurring carbonate mineral phases are calcite, aragonite, and magnesium calcite (Mg-calcite). Benthic organisms produce aragonite and calcite, which have the same chemical composition but a different mineral structure (orthorhombic versus rhombohedral), whereas calcite and Mg-calcite have the same structure but calcium ions have been randomly replaced by magnesium ions (up to ~30 mol%). Calcite is the most stable form o the three. Andersson et al. (in press) performed a synthesis of the literature regarding the response of benthic calcifiers to ocean acidification. This synthesis presents the state of our knowledge of impacts of ocean acidification on marine biogeochemical cycles, interaction with climate change and feedbacks to the earth system.

Thermodynamic principles suggest that as seawater carbonate ion concentration and carbonate mineral saturation state (!) decrease as a result of pH decline, one would expect that the rate of calcification of benthic marine calcifiers would decrease. Most data are consistent with a decline in the rate of benthic calcification as a result of ocean acidification, although few recent studies show no response or an increase in calcification in a range of different benthic calcifiers exposed to moderately elevated CO_2 conditions. However, regardless of whether calcification changes in marine organisms in response to elevated CO_2 and lower !, deposition of $CaCO_3$ is thermodynamically less stable under such conditions. It has been argued that some organisms may be able to up-regulate their metabolism including calcification to compensate for increased acidity of seawater. Based on this synthesis approach, it is concluded that benthic biogeochemical processes such as calcification and $CaCO_3$ dissolution as well as the community composition of benthic ecosystems will most likely be significantly altered by OA.

2.4 Synthesis of the literature pertaining to the response of benthic calcification (Partner 1.1: LOV).

The consequences of ocean acidification to benthic organisms and ecosystems could be significant but are, overall, poorly known and quantified at this time. By integrating the current knowledge on the effects of ocean acidification on major benthic biogeochemical processes, individual benthic organisms, and observed characteristics of benthic environments as a function of seawater carbonate chemistry, it is possible to draw some general conclusions regarding the response of benthic organisms and ecosystems to a world of increasingly higher atmospheric CO_2 levels. Large-scale geographical and spatial differences in seawater carbonate system chemistry, owing to both natural and anthropogenic processes, provide a powerful means to evaluate the effects of ocean acidification on marine benthic systems. Based on this approach, it is concluded that benthic biogeochemical processes such as calcification and $CaCO_3$ dissolution as well as the community composition of benthic ecosystems will most likely be significantly altered by ocean acidification. (Andersson et al., 2011).

3. PTEROPODS

3.1 Impact of ocean acidification on calcification of Mediterranean and Arctic pteropods (Partner 1.1: LOV)

A strong relationship between the aragonite saturation state (Ωa) and the calcium carbonate precipitation rate was found for both Arctic and Mediterranean pteropods. Dissolution of the shell in undersaturated conditions has also been reported on adult and juvenile Mediterranean pteropods.

Larvae of the Mediterranean pteropod Cavolinia inXexa were maintained at controlled pH_T values of 8.1, 7.82 and 7.51, equivalent, respectively, to pCO_2 levels of 380, 857 and 1,713 µatm. At pH_T 7.82, larvae exhibited malformations and lower shell growth, compared to the control condition. At pH_T 7.51, the larvae did not make shells but were viable and showed a normal development. However, smaller shells or no shells will have both ecological (food web) and biogeochemical (export of carbon and carbonate) consequences. These results suggest that pteropod larvae, as well as the species dependent upon them or upon adults as a food resource, might be significantly impacted by ocean acidification (Comeau et al., 2010a).

Thecosome pteropods (pelagic mollusks) can play a key role in the food web of various marine ecosystems. They are a food source for zooplankton or higher predators such as fishes, whales and birds that is particularly important in high latitude areas. Since they harbor a highly soluble aragonitic shell, they could be very sensitive to ocean acidification driven by the increase of anthropogenic CO2 emissions. The effect of changes in the seawater chemistry was investigated on Limacina helicina, a key species of Arctic pelagic ecosystems. Individuals were kept in the laboratory under controlled pCO2 levels of 280, 380, 550, 760 and 1020 µatm and at control (0°C) and elevated (4°C) temperatures. The respiration rate was unaffected by pCO2 at control temperature, but significantly increased as a function of the pCO2 level at elevated temperature. pCO2 had no effect on the gut clearance rate at either temperature. Precipitation of CaCO3, measured as the incorporation of 45Ca, significantly declined as a function of pCO2 at both temperatures. The decrease in calcium carbonate precipitation was highly correlated to the aragonite saturation state. Even though this study demonstrates that pteropods are able to precipitate calcium carbonate at low aragonite saturation state, the results support the current concern for the future of Arctic pteropods, as the production of their shell appears to be very sensitive to decreased pH. A decline of pteropod populations would likely cause dramatic changes to various pelagic ecosystems (Comeau et al 2010b).

***3.2 p*CO_2 effects on pre-winter and overwintering juvenile pteropods in the Arctic.** (Partner 3: IFM-Geomar). During fall 2009 perturbation experiments with the polar thecosome pteropod *Limacina helicina* and in winter 2010 with *L. helicina* and the boreal-atlantic *L. retroversa* were carried out in Ny Ålesund (Svalbard).
In fall 2009, temperature and *p*CO_2 were shown to have a significant effect on mortality, but temperature was the overriding factor. Shell diameter, shell increment and shell degradation were significantly impacted by *p*CO_2 but no significant effect for temperature could be shown (Lischka et al. 2011). Moreover, no synergistic effects could be shown. Preliminary results from ^{13}C incubations showed trends of ^{13}C enrichment in organisms with increasing temperature and decreasing enrichment with increasing *p*CO_2 levels, suggesting a positive temperature and a negative CO_2 effect on pteropod calcification rates.
Our winter investigations proved that both *Limacina* species cease growth during their overwintering period, i.e. no natural calcification occurs. Rising *p*CO_2 levels negatively affected shell degradation, i.e. dissolution of *L. helicina* and *L. retroversa*, but the shells of atlantic *L. retroversa* were affected stronger. Moreover we found temperature and CO_2 acting synergistically to enhance the shell degradation in the polar *L. helicina* at higher levels whereas temperature did not amplify dissolution in *L. retroversa*. Compared to our fall results where we did not find any significant synergistic effects, this suggests a seasonal effect with higher sensitivity to these stressors during winter.
During both seasons, evidence for active counteraction against dissolution was found suggesting that pre-winter and overwintering pteropods may be constrained to raise their cost of maintenance and use up energy reserves that are stored for the continuation of their development in spring and reproduction. Due to *Limacina's* winter phenology as well as the specific carbonate chemistry (change) characteristics of Arctic (winter) waters, however, our results strongly suggest that negative climate change effects on Arctic thecosome pteropods will first show up during winter, possibly before ocean acidification effects in summer will become a serious threat.

4. FORAMINIFERA

4.1 Investigation of the vesicle dynamics in foraminifera (Partner 5:AWI).
The calcium carbonate produced by foraminifera is a source of information for paleoceanographical studies. To optimize the calibration of proxies it is very important to identify the physiological processes transporting the ions from the source (seawater) into

the foraminiferal calcite. A special fluorescent dye, FITC-dextran (Fluoresceinisothiocyanato-dextran), was used to study the uptake of seawater into the cell and its sub-sequent fate. The results give us new insights into the calcification process, suggesting that a commonly accepted endocytosis-based calcification model (Erez, 2003) cannot be applied to *Ammonia tepida*, because seawater endocytosis is not related to chamber formation.

4.2 Magnesium incorporation into shells of *Orbulina universa* (Partner 5: AWI)
The incorporation of magnesium (Mg) into the foraminiferal shell is very sensitive to calcification temperature. However, it has been shown recently that the Mg/Ca ratio of seawater also influences the amount of Mg incorporated into foraminiferal tests. Therefore foraminiferal Mg/Ca ratios also reflect past seawater Mg/Ca ratios which changed throughout Earths' history. Eggins (unpublished data) has shown experimentally on one species, *Orbulina universa*, that the Mg/Ca thermometer may have been less sensitive in past oceans, when Mg/Ca < ambient. This implies that Mg/Ca variations in ancient oceans could reflect larger temperatures than currently calculated. In order to confirm this hypothesis we cultured a different planktonic species (*G. ruber*) at two different temperatures under a range of three Mg/Ca ratios at the IUI in Eilat. Since Mg/Ca thermometry is widely used in paleooceanography this study will add value to deep-sea core-derived palaeoceanographic and climate records. First results indicate that Mg/Ca ratio in the shell increases with increasing Mg/Ca ratio in the seawater as expected but, that the distribution coefficient is not a constant and hence compromises the Mg/Ca paleothermometer.

4.3 Mg and Sr distribution in the shell of *Ammonia tepida* (Partner 5: AWI)
Trace element, e.g. magnesium and strontium, to calcium ratios in foraminiferal shells are widely used as proxies, i.e. stand-in variables, for environmental parameters (the so-called target variable). The calibration of these proxies relies on empirical relationships providing the algorithm to convert proxy values to target values. In practice it is often difficult to apply a given proxy, because the latter is, more often than not, subject to secondary influences, i.e. environmental parameters other than the target variable. A reliable interpretation of proxy signals, therefore, requires an understanding of the physiological and inorganic processes leading to incorporation of trace elements into foraminiferal shells. One issue of relevance in this context is the distribution of these trace elements within the shell. While an even distribution of trace elements would suggest that one set of processes is always employed in the same way, an uneven distribution would indicate discrete phases of shell formation governed by different, or a different combination of, processes. We measured distribution maps of Mg/Ca, Sr/Ca, and Mg- and Ca-isotopes in cross-sections of *A. tepida* shells cultured at a range of Mg/Caseawater and Sr/Caseawater by means of NanoSIMS. We found Mg and Sr rich areas (bands), the Mg-bands being in the same place where the Sr-bands were detected. Mg- and Ca-isotopes were evenly distributed.

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