News and views on mitochondrial water transport

Patrizia Gena1,2, Elena Fanelli1, Catherine Brenner3, Maria Svelto1, Giuseppe Calamita1

1Dipartimento di Fisiologia Generale ed Ambientale, Università degli Studi di Bari, Bari, Italy, 2Department of Physiology, Johns Hopkins University, Baltimore (MD), USA, 3Université de Versailles-St Quentin, PRES UniverSud Paris, CNRS UMR 8159, Versailles, France

1. ABSTRACT

The osmotic movement of water into and out of the mitochondrial matrix underlies the extraordinary plasticity that characterizes mitochondria, a feature of pivotal importance to cell bioenergetics and signaling, and of critical relevance to life-and-death cell decision. However, the biophysics and identity of mitochondrial water transport had remained mostly unexplored, until recent works suggesting high water permeability and the presence of multiple facilitated pathways of water diffusion in liver mitochondria. Here, we attempt to summarize our current view of the mechanisms of mitochondrial water transport and possible relevance of the channel-mediated pathways created by mitochondrial permeability transition, aquaporins and protein/lipid specializations. Assessing the molecular bases and dynamics of mitochondrial water permeability will help to answer the much-debated question over the role of mitochondria in apoptotic and necrotic cell death.

2. WATER TRANSPORT IN MITOCHONDRIAL VOLUME HOMEOSTASIS

The osmotic movement of water accompanying solutes between the cytoplasm and the mitochondrial matrix is fundamental to mitochondrial volume homeostasis, a housekeeping function underlying the striking shape and volume plasticity characterizing the organelle and of pivotal relevance to essential biological functions. Indeed, remarkable changes in mitochondrial volume are associated both with physiological processes such as oxidative phosphorylation, intracellular signal transduction and mitochondrial fusion and fission, and patho-physiological conditions such as those occurring in liver regeneration, reactive oxygen species (ROS) overproduction and ischemia/reperfusion cell injury (see 1) for a Review). Loss of mitochondrial volume homeostasis is often accompanied with the remodelling of cristae through cleavage ofOPA1, a dynamin-related GTPase regulating mitochondrial fusion, and presenilin-
Mitochondrial water transport

associated rhomboid-like (Parl), a rhomboid protease (2), and swelling of the organelle being the consequence of a variety of reasons, from modulation in solute channels and exchanger functions to uncontrolled fluid movement into the mitochondrial matrix (see (3) for Review). Heterogeneity in organelle size and shape have been reported in patients with point mutations in mitochondrial DNA (4) while rearrangements of mitochondrial morphology associated with oxidative phosphorylation (OXPHOS) defects have been suggested to be secondary to alterations of non-mitochondrial cellular functions (5). Steric hindrance of swollen mitochondria may have a series of repercussions on cellular functions, such as that of altering the trafficking of mitochondria to cellular destinations where ATP synthesis is required (6) or impairing the transport of organelles, as suggested for seamless axonal transport in cerebellar granule neurons (7). A rather unexpected action is the one suggested in cardiomyocytes where mitochondrial swelling has been hypothesized to impose mechanical constraints inside the cell, leading to an increase in the force developed by myofibrils (8). Osmotic swelling has also been suggested to lead to breakdown of the mitochondrial reticulum with consequent selective mitochondrial autophagy or mitophagy (9). More in general, mitochondrial swelling is not simply the final stage of mitochondrial dysfunction, but plays a crucial role in cell injury and death. Indeed, mitochondrial swelling is one of the main mechanisms by which cytochrome c, apoptosis inducing factor (AIF) and other procaspases are released into the cytosol, leading to apoptotic cell death (10, 11).

Mitochondrial volume changes are modulated mainly by the net movement of solutes, including K+ and Ca2+ ions, across the inner mitochondrial membrane (IMM), the major barrier for solutes moving between the cytoplasm and the mitochondrial matrix (3, 12), and a number of ion channels and exchangers have been identified and characterized for their ability to transport solutes across the mitochondrial membranes (see (13-15) for Reviews). Although high water permeability in mitochondria had already been reported more than twenty years ago, as a feature for keeping mitochondria in a state of osmotic equilibrium with their environment (16), the topic of mitochondrial water pathways and biophysics was boosted just recently, after reporting immunoreactivity to aquaporin water channels and suggesting the existence of facilitated pathways of water transport in mitochondria (17-23). Both aquaporins and high water permeability in mitochondria were debated in a recent study by Yang and coworkers (24). The present Review will discuss the most recent results in terms of mechanism, molecular identity and relevance of mitochondrial water transport and express our current view on some debated questions and controversial issues. In this study, we will not analyze the role of mitochondrial ion transport and membrane potential in mitochondrial volume homeostasis, which is extensively discussed elsewhere (3, 11-13, 25-28).

3. STRUCTURAL, BIOPHYSICAL AND TECHNICAL CONSTRAINTS IN THE ASSESSMENT OF MITOCHONDRIAL WATER PERMEABILITY

Although mitochondria are well-behaved osmometers (16), studying mitochondrial water transport is a rather complicated task, due to a series of structural, biophysical and technical constraints.

First of all, mitochondria are organelles bounded by two membranes, the outer (OMM) and inner (IMM) membranes, that constitute discontinuous barriers in series for water moving into and out of the matrix. While the OMM is not considered of major resistance to transport, due to the voltage dependent anion channel (VDAC), a large diameter channel (≈ 3 nm) that is permeable to uncharged molecules up to ≈ 5 kDa (29), the IMM is believed to represent the main barrier for molecules moving between the cytoplasm and the matrix. IMM is composed of an inner boundary (part adjacent to the OMM) and crystal portions (30) that are considerably different in terms of intrinsic composition, function and permeability. Although the crystal portion is the much larger one, the ratio between the two portions is a dynamic one, depending on the functional state of the mitochondrion (12, 16). Stunning changes in terms of mitochondrial permeability occur as a consequence of opening the mitochondrial permeability transition pore (PTP), a non-specific pore across the mitochondrial membranes (see (31) for a recent Review). While impermeable to all but a few selected metabolites and ions, mitochondria become leaky (cut-off of about 1.5 kDa), uncoupled and massively swollen when exposed to high Ca2+ concentrations, especially in the presence of phosphate and when accompanied by oxidative stress. Conversely, some conditions, such as matrix acidification and high concentrations of ADP and Mg2+, are known to potently inhibit pore opening in situ (32). PTP is kept closed with transient openings under normal physiological conditions and experiences long-lasting and presumably irreversible openings under pathological conditions (33; for a recent Review, see (34)).

The accuracy of mitochondrial water transport measurements may be considerably affected by technical constraints. Isolating mitochondria in potassium-free sucrose media may provoke membrane damage and disruption of membrane contact sites with consequent matrix contraction (12). This is a very critical aspect when using stopped-flow light scattering, a technique linking light transmission to mitochondrial volume changes,
Mitochondrial water transport
to measure the water permeability of suspended whole mitochondria. While applicable for assessing the osmotic water permeability of mitochondrial membrane vesicles (18, 22, 24), light scattering cannot provide information on mitochondria in situ, within the cytoplasm. Although of direct application when defining the rate constant of mitochondrial shrinkage/swelling (Kᵣ), a biophysical parameter providing limited information on water transport, stopped-flow light scattering can only lead to a calculation of the mitochondrial osmotic water permeability coefficient, Pₛ, once the surface-to-volume (S/V) ratio of the specimen analyzed is known (35). Unlike spherical specimens, for which the radius is sufficient to calculate surface area and volume, the S/V ratio becomes difficult to determine when dealing with rod-shaped structures like mitochondria (6, 36). While traditional light (confocal fluorescence) and electron microscopy techniques used to measure the mitochondrial volume have been argued to present several disadvantages (for a Review, see (3)), a new approach combining confocal microscopy with 3D deconvolution analysis was recently set up to quantify more accurately the volume and other morphological parameters of mitochondria (37).

4. THE HIGH WATER PERMEABILITY AND PRESENCE OF AQUAPORINS IN MITOCHONDRIA IS THE OBJECT OF MUCH DEBATED

Due to their small size, mitochondria have a high S/V ratio (mean value of ≈500 cm²/µl of matrix volume), roughly two orders of magnitude greater than that of whole cells. Hence, they are able to reach osmotic equilibrium in tens of milliseconds in response to osmotic gradients. However, although no doubts exist as to the fact that simple diffusion of water across the OMM and IMM lipid bilayers is sufficient to ensure organelle osmotic homeostasis, recent observations suggest the existence of channel-mediated pathways (facilitated diffusion) in addition to the lipid pathway (17-19, 22). By stopped-flow light scattering, both intact whole mitochondria and related membrane subcompartments showed very high osmotic water permeability (Pₛ values ranging among 300 and 500 µm/s at 20 °C; (18, 22)). The osmotic water permeability of OMM and IMM vesicles was surprisingly quite comparable, since the outer membrane is commonly considered a relatively minor barrier (i.e. compared to the IMM) to solutes moving into and out of the mitochondrial matrix (29). As previously discussed (22), this unexpected result may be relevant to the opening state of VDAC, a possibility that needs to be specifically addressed. Based on their centrifugal behavior, the highest values of Pₛ were measured in IMM vesicles prepared from the heaviest mitochondria (1000xg gravitational fraction), namely the mitochondrial subpopulations having the largest diameter and highest respiration rate, O₂ uptake and respiratory control ratios (RCRs) when compared to lighter mitochondria subpopulations (38, 39). The hypothesis of channel-mediated water transport pathways was also supported by at least three other arguments. First, the Arrhenius activation energy (E_a) associated with the osmotic flux of water across mitochondrial membranes was low (3.93 kcal/mol) and consistent with facilitated water diffusion (18, 22). The fact that lipid mobility is greater at increased temperatures explains why pore-mediated water transport is relatively unaffected by temperature changes (E_a < 6 kcal/mol) compared to diffusional movement through the membrane lipids (E_a > 10 kcal/mol). Second, the Pₛ of the IMM vesicles resulted eight times higher than that of protein-free IMM liposomes (451 ± 52 and 57 ± 6 µm s⁻¹, respectively; (22)). Third, the mitochondrial osmotic water permeability was partially inhibited after treatment with Hg²⁺ (18, 22), Ag⁺ and Cd²⁺ (19) ions, sulfhydryl reagents known to block the movement of water across proteinaceous channels with cysteine residues in the vicinity of the aqueous pores. Inhibition of mitochondrial water transport by Hg²⁺ was also seen by Yang and coworkers who claimed that the slower rate of osmotic equilibration (Pₛ reduction) could be accounted for quantitatively by altered vesicle size rather than reduced intrinsic water permeability (24). This is a questionable assertion, because no significant changes to vesicle diameter with IMM and OMM vesicles exposed to the Hg²⁺ ion were observed in our laboratory, where vesicle morphometry was evaluated both by electron microscopy and instrumental size analysis (22). Interestingly, additional pathways of facilitated water conductance other than the Hg²⁺-sensitive one was suggested by the fact that the Arrhenius activation energy of Hg²⁺-treated mitochondria was low, being still in line with channel-mediated transport (18). Aquaporin-8 water channel (AQP8) immunoreactivity was found in the IMM of liver (17, 18), kidney cortex (19) and other organs (17, 21), observations that led to the tempting hypothesis that AQP8 could be one of the predicted mitochondrial water channels. However, subsequent studies reporting upregulation of liver mitochondrial AQP8 by the triiodothyronine hormone showed no differences in terms of osmotic water permeability between the liver IMM vesicles from rats in the various thyroid states (40). This was also consistent with another study showing that the overall water permeability of brain mitochondria that lack AQP8 water channels corresponds to that of liver and testis mitochondria that, by contrast, have AQP8 in their IMMs (22). Although mitochondrial AQP8 has also been speculated to be functionally relevant to the urea cycle (22, 41), cell-to-cell signalling and reactive oxygen species (ROS) generation (42), its significance remains open to question and a matter for further consideration, given that aquaporins are being found to play a large (and unexpected) number of physiological functions (18, 43). It is reasonable to think that, as AQP8 immunoreactivity appears to be heterogeneously distributed among mitochondria.
Mitochondrial water transport

(18), precise data on the functional relevance of AQP8 in mitochondria may come from the isolation and biophysical analysis of the mitochondrial subpopulations containing "physiological" levels of AQP8. Both the functional meaning of mitochondrial AQP8 and the high osmotic water permeability of liver mitochondria are argued by Yang and coworkers who, based on their biophysical study reporting fairly high intrinsic membrane water permeability (Pf of 90 μm/s at 10 °C) for liver mitochondria, claimed that the absence of significant AQP8 immunoreactivity in mitochondria is consistent with the high mitochondrial S/V ratio producing millisecond osmotic equilibration (24).

An alternatively spliced isoform of AQP9, an aquaglyceroporin permeable to water, glycerol and a series of small solutes, was reported to be expressed in the IMM of astroglia throughout rat brain and a subset of neurons (20). However, significant AQP9 immunoreactivity in mouse brain was not confirmed by a further work using AQP9-/− knock-out mice (44).

The meaning of the presence of aquaporins (or, more in general, facilitated pathways for water diffusion) in organelles with a high S/V ratio (i.e., mitochondria, endoplasmic reticulum and secretory vesicles) is currently the object of intense debate (43, 45, 46). The known poor availability of aquaporin antibodies, including those against AQP8 and AQP9, should not be ignored in explaining conflicting immunoreactivity data. Provocatively, osmotic sensation and water transport in controlled vesicle swelling have been hypothesized to explain the functional relevance of aquaporins in intracellular organelles with high S/V ratios such as mitochondria (45) and secretory granules (46, 47). Concerning the extent of mitochondrial osmotic water permeability, a critical aspect in predicting pathways of facilitated water transport in mitochondria, the discrepancies between our data (18) and those reported by the Verkman laboratory (24) may reflect the limitations of the structural relationship existing between light scattering and mitochondrial matrix volume (12, 48, 49). Careful analysis of osmotic equilibrium curves obtained with mitochondria exposed to a series of osmotic gradients showed that the linearity between light scattering and matrix volume may deviate, depending specifically on the initial extent of matrix volume and not on osmolality per se (16). Moreover, isolation artefacts may occur following prolonged incubation of mitochondria in K+ free medium with consequent loss of matrix K+ (via K+/H+ exchange) and anions. This causes the matrix to become highly contracted and the intermembrane space to undergo a drastic increase in volume (12, 50). It is reasonable to think that loss of solutes due to isolation artefacts may affect the stopped-flow light scattering analysis of mitochondria exposed to osmotic upshift, with consequent underestimation of the osmotic water permeability.

5. CLASSICAL AND NON-CLASSICAL PERMEABILITY TRANSITION PORES AND MITOCHONDRIAL WATER PERMEABILITY: IS THERE ANY RELATIONSHIP?

The large pore formed by PTP across the mitochondrial membranes is a good candidate to explain the facilitated pathway underlying the high water permeability featured by mitochondria. However, although the opening of PTP is postulated to mediate influx of water (together with solutes) causing mitochondrial-matrix swelling (25, 51, 52), no experimental work has been performed to evaluate biophysically such a hypothesis. The question was addressed in a recent study by our laboratory showing major changes in water permeability in rat liver mitochondria exposed to known modulators of the opening/closure of the PT pore (Fanelli et al, manuscript in preparation) and supporting the idea of a functional involvement of PTP in mitochondrial water transport. Since in the absence of apoptotic stimuli, the PT pore flickers in a low-conductance state at a frequency determined by matrix-free [Ca2+] (53-56), it is tempting to think that an important share of the overall mitochondrial water permeability is sustained by PTP. This would be also consistent with the long-standing idea (25, 51) that, in apoptotic conditions, the prolonged opening of PTP following pro-apoptotic signals may mediate massive movement of water and small solutes into the mitochondrial matrix causing mitochondria to swell and release cytochrome c and other pro-caspase factors. Entry of water into the matrix may either be driven by the colloidal osmotic pressure exerted by matrix proteins that remain impermeable (57), or else be the result of a large potassium influx down to the concentration gradient, as recently suggested by the Kaasik laboratory (3).

Because sensitivity to cyclosporin A (CsA), an immunosuppressant agent specifically blocking the opening of PTP, is considered a defining feature of the "classical" PTP, the so-called "non-classical" permeability transition is characterized by being CsA-insensitive. A previous study of osmotic swelling by Lee and coworkers using proximal tubule cells treated with Cd2+ concentrations to induce apoptosis suggested AQP8 as an important CsA-insensitive pathway underlying mitochondrial permeability transition (19). At micromolar cytoplasmic concentrations, the Cd2+ ion was speculated to provoke mitochondrial swelling by interacting with the IMM Ca2+ uniporter and AQP8 water channels. However, there is no evidence that AQP8 is activated by Cd2+ (or other divalent cations such as Ca2+) and, as already discussed (section 4), the water channel relevance of AQP8 in mitochondria is an issue open to question (24).

Both the molecular mechanism and identity of the CsA-insensitive PT pores remain poorly
characterized (58), a limitation affecting studies on the potential water conductance activity of the related megachannels. One CsA-insensitive PT pore is PalCaP, a non-specific channel of lipid nature described by Sultan and Sokolove after observing that palmitic acid promotes a non-classical increase in permeability transition that is clearly distinguishable from the occurrence of the classical mitochondrial PT (59). PalCaP was reported to close spontaneously after opening, whereas its prolonged open state was suggested to lead to permeability transition (59-61). Subsequent investigation should be addressed to other known non-classical mitochondrial PT pores as potential facilitators of water transport. Among these, 1) the “ceramide channel”, a large pore induced by the pleiotropic lipid messenger ceramide that regulates a diverse range of cellular processes, including apoptosis, cell growth, and differentiation (62, 63), 2) the ATP/ADP translocator (ANT) permeability transition pore induced by arachidonic acid (64), 3) the non-classical PT due to the conductive pore formed by the mitochondrial protein import machinery at the level of the outer and inner mitochondrial membranes (65-67), and 4) PT pores formed by the aggregation of misfolded integral membrane proteins damaged by oxidant and other stresses (58). Regarding the latter PT pores, it is reasonable to hypothesize that chaperone-like proteins initially block conductance through misfolded protein clusters and that increased Ca++ opens these regulated PT pores, an effect blocked by CsA. However, when protein clusters exceed chaperones available to block conductance, unregulated pore opening would occur. Finally, members of the Bcl-2 family such as Bax or Bak have been reported to form very high-conductance channels when reconstituted in liposomes, an observation that has led to the hypothesis that these pro-apoptotic proteins form megachannels across the OMM (68). Following apoptosis induction by Bax and Bak (but not Bid and Bad), Bax leaves the mitochondrial membranes and coalesces into large clusters containing thousands of Bax molecules that remain adjacent to the mitochondria. The formation of these clusters does not require caspase activity, but is completely and specifically inhibited by Bcl-XL, demonstrating their relevance to the apoptosis process (69).

6. WORKING MODEL OF THE MOVEMENT OF WATER ACROSS MITOCHONDRIAL MEMBRANES

According to the available information and our current view, a working model accounting for high mitochondrial water permeability can be attempted (Figure 1). Driven by an osmotic gradient created by the net movement of solutes (i.e., K+ and Ca2+ ions), water may flow by simple diffusion into and out of the mitochondrial matrix by moving through the lipid bilayers of the outer and inner membranes and the rather hydrophilic domain created by the exceedingly high amount (76%) of proteins composing the IMM. Depending on the (dys-)functional state of mitochondria, facilitated diffusion of water may occur through the channels formed by VDAC (OMM), AQP8 (IMM) and permeability transition pores (OMM plus IMM). The contribution provided by PTP to water permeability may depend
Mitochondrial water transport

on the mitochondrial functional states. While in normal conditions (absence of apoptotic and necrotic stimuli), the Ca2+-dependent flickering of PTP in its closed/low conductance state would provide a major contribution to the basal mitochondrial water permeability by preserving the organelle structural integrity, the long-lasting opening of the PTP occurring in pathological conditions would mediate massive movement of water (and solutes) into the matrix with associated swelling and rupture of the organelle and consequent cell death. However, the possibility that water moves directly through the megachannels formed by VDAC and PTP needs to be verified experimentally.

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Regulated water transport is of critical importance for mitochondrial volume homeostasis, a housekeeping function thought both to help regulate biological processes such as oxidative capacity, ROS production, cytochrome c release and mechanical signaling, and to maintain the structural integrity of the organelle. As a matter of fact, uncontrolled entry of water into the matrix compartment causes mitochondria to swell, leading to deleterious consequences that can culminate in cell death (70).

Although high membrane water permeability is often synonymous with diffusion of water across facilitated pathways, the biological need for water-conducting channels in organelles with a high S/V ratio such as mitochondria remains the object of debate (24). However, an undeniable feature associated with channel-mediated molecular transport is that it can be regulated. Aspects that cannot be ignored in explaining the discrepancies raised when addressing the water permeability of mitochondria experimentally are the (i) non-negligible structural constraints presented by the organelle, (ii) limited linearity of the relationship between light scattering and mitochondrial volume changes, and (iii) possible artifacts associated with their isolation. It is likely that existing inconsistencies will be solved after standardizing the experimental procedures used for the biophysical characterization of the organelle.

The water channel activity of AQP8 may be only relevant to the water permeability of given subpopulations of liver mitochondria (22), a fact that may explain why overall osmotic water permeability in AQP8-/− mouse mitochondria appears to be of the same magnitude as in wild-type mice (24). Valuable information should come from the biochemical and biophysical analyses of the (isolated) subpopulations of mitochondria expressing aquaporin-8 at “physiological” levels. Although mitochondrial AQP8 has been speculated at various levels to be relevant to the urea cycle (22, 41), cell-to-cell signalling, ROS generation (42), or act as an osmotic sensor (45), the fact that it is absent in the mitochondria of immortalized mouse hepatocytes (no apoptosis) and rat spermatozoa (no βeta-oxidation of fatty acids) (18) provides useful clues for future functional studies.

Our most recent biophysical work suggesting involvement of PTP in mitochondrial water transport is an important finding, supporting the long-standing postulation that, in the open state, such large non-selective pores mediate the entry of water and solutes into the matrix (57). However, whether PTP conducts water directly through the megachannel formed by its multiprotein complex or, alternatively, (by transporting small solutes) creates the osmotic conditions for the movement of water through other neighborhood pathways, remains to be assessed. Our preliminary evidence suggesting CsA-insensitive pathways accounting for a non-negligible share of mitochondrial water transport suggests potential involvement of non-classical PT pores in mitochondrial water permeability, a matter deserving further investigation.

Unraveling the precise molecular identities and biophysics of mitochondrial water transport will undoubtedly facilitate both our knowledge of the mechanism of mitochondrial volume homeostasis and our understanding of the role played by mitochondria in critical processes such as apoptotic and necrotic cell death.

8. ACKNOWLEDGMENTS

Financial support from MUR (Ministero dell’Università e della Ricerca, grant PRIN-Cofin 2006 to G.C.), Università Italo-Francese (Progetto Galileo to G.C. and C.B.), University of Bari (grant “Fondi di Ateneo per la Ricerca Scientifica” to M.S. and G.C.), OSEO-ANVAR (to C.B.), Association pour la Recherche sur la Cancer (ARC; to C.B.) and Institut National du Cancer (INCa; to C.B.) are gratefully acknowledged.

9. REFERENCES


4194
Mitochondrial water transport

fibroblasts of patients with point mutations in mitochondrial DNA. *Ultrastruct Pathol*, 30, 239-245 (2006)


Mitochondrial water transport


49. Das, B. & C. Sarkar: Mitochondrial K ATP channel activation is important in the antiarrhythmic and cardioprotective effects of non-hypotensive doses of nicorandil and cromakalim during ischemia/reperfusion: a study in an intact
Mitochondrial water transport


**Abbreviations:** ROS, reactive oxygen species; Parl, presenilin-associated rhomboid-like; OPA1, Optic atrophy protein 1; AIF, apoptosis inducing factor; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; VDAC, voltage dependent anion channel; PTP, permeability transition pore; RCRs, respiratory control ratios; AQP8, Aquaporin-8; AQP9, Aquaporin-9; CsA, cyclosporin A; ANT, adenine nucleotide translocase; Bcl-2, B-cell leukemia/lymphoma 2; Bax, Bcl-2-associated X protein; Bak, Bcl-2-antagonist/killer 1; Bid, BH3 interacting domain; Bad, Bcl-2-associated death promoter; CypD, cyclophilin D; IMS, intermembrane space
Mitochondrial water transport

**Key Words:** Mitochondrial Swelling, Water Transport, PTP, Aquaporin, Apoptosis, Necrotic Cell Death, Review

**Send correspondence to:** Giuseppe Calamita, Dipartimento di Fisiologia Generale ed Ambientale Universita degli Studi di Bari. Via Amendola, 165/A. 70126 Bari, Italy, Tel: 39 0805442928, Fax: 39 0805443388, E-mail: calamita@biologia.uniba.it