Int Aquat Res (2022) 14:231–240
https://doi.org/10.22034/IAR.2022.1965012.1317

REVIEW

Present and future opportunities in the use of atomic force microscopy to address the physico-chemical properties of aquatic ecosystems at the nanoscale level

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Received: 07 August 2022 / Accepted: 25 November 2022 / Published online: 30 November 2022

Abstract Microorganisms interplay with their neighbors according their intrinsic cellular adhesion and membrane rigidity. The protein receptors located at the cellular membrane mediate specific recognition processes with biomolecules and others cells. This topic has generated great interest based on the huge variability displayed at different environmental conditions. Aquatic ecosystems present notably versatility of microorganisms and dissolved molecules that mutually influence in their viability, proliferation and biological performance. Biodiversity preservation of the current water reservoirs strongly depends on the gathering of these properties and their accurate interpretation. Bulk measurements from meso-scale techniques provide average information of aquatic microorganisms hidden relevant details. For this reason, it is opportune to devote high-throughput single molecule tools as atomic force microscopy (AFM) to determine the physico-chemical properties exhibited by individual aquatic microorganisms and thus, establish a full comprehension of all parameters that influence the state of aquatic ecosystems. The present work aims to highlight the potential applications and future perspectives of AFM for the study of all living entities involved in aquatic ecosystems to better understand their inherent characteristics at the single molecule level.

Keywords Aquatic ecosystems . Atomic force microscopy (AFM) . Force spectroscopy (FS) . Nanoindentation . Nanoscale . Single molecule biophysics . Young's modulus

Introduction

Today, Earth faces numerous challenges to preserve the survival of the living beings that inhabit it. One of the most critical points is to safeguard the integrity of aquatic ecosystems due to their regulating activity in terms of pH, temperature, and marine biodiversity (Hosseini et al. 2017). Aquatic ecosystems are divided in wetlands, rivers, lakes and coastal estuaries. This homeostasis is especially important because the eutrophic microorganisms which live in aquatic ecosystems allow the uptake and subsequent fixation of atmospheric carbon dioxide, CO_2 (Prasad et al. 2021; Iglina et al. 2022). Aquatic microorganisms are a double-edged sword to water quality. First, their function in the bioremediation of chemical hazards and nutrient cycling significantly improve the water quality and maintain the good health of aquatic ecosystems. For example, microalgae and cyanobacteria have shown to be efficient organisms to remove harmful compounds like pesticides coming from farms (Castellanos-Estupiñán et al. 2022) and heavy metals produced by industrial activity (Ankit et al. 2022), respectively. Moreover, the decrease in aquatic animal and plant richness may lead the loss of opportunities to find novel compounds with currently unknown implementations. Two re-

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Table 1 Summary of techniques for addressing the physico-chemical properties of aquatic ecosystems addressed in this review

Technique	Spatial resolution	Staining/ Labeling	Measurements at room temperature	Measurements in liquid media	Fixation on surface
AFM	1 nm	No	Yes	Yes	Yes
SEM	0.6 nm	Yes	Yes	No	Yes
Cryo-TEM	0.2 nm	Yes	No	No	Yes
STED	30 nm	Yes	Yes	Yes	Yes

cent examples from non-aquatic organisms are the following: I) substances excreted by the rosy periwinkle from Madagascar which are currently being studied for leukemia treatments in children (Caputi et al. 2018). II) Peeling bark from the Pacific yew synthetizes taxol. Taxol is a molecule which has been reported to be effective against ovarian, breast and esophageal cancers or Kaposi's sarcoma (Weaver 2014). The aforementioned examples illustrate the high-value of aqueous ecosystems and their potential future importance by finding so far unknown biomolecules that may act as therapeutic agents against human malignancies of currently unknown cure, such as cancer, neurodegenerative disorders (e.g. Alzheimer's, Parkinson's or Huttington's diseases), among others (Ghoran and Kijjoa 2021). For these reasons, the preservation of wildlife is of crucial importance to ensure human future welfare. Therefore, governs and society stakeholders have made great efforts to investigate the associate mechanisms of microoganisms living in aquatic ecosystems and their physico-chemical properties. In this framework, nanotechnology can play a central core to address the properties and nature of aquatic living microorganisms. Nanotechnology tools like atomic force microscopy (AFM) can act as one of the main key actors to shed light of many remaining questions linked to living organisms from aquatic ecosystems. AFM has emerged as feasible technique to determine numerous soft matter properties like the morphology of cells (Garcia-Diosa et al. 2021) and biomolecules (Vega et al. 2013) by AFM imaging, the adhesion forces of cells (Shinde et al. 2021) and biomolecules (Marcuello et al. 2012) by force spectroscopy (FS), the chemistry of lipid subcellular reorganization of *Parachlorella kesslerii* microalgae (Deniset-Besseau et al. 2021) and amyloidogenic proteins from Archaea (Otzen et al. 2021) by AFM-nanoscale infrared spectroscopy (AFM-nanoIR), magnetic behavior of *Magnetospirillum* magnetotactic bacteria by magnetic force microscopy (MFM) (Marcuello et al. 2018), electrochemical response of viral nanoparticles by AFM-scanning electrochemical microscopy (AFM-SECM) (Paiva et al. 2022) or mechanical parameters of biopolymers (Marcuello et al. 2020) by nanoindentation, among others. AFM presents many advantages in comparison to other single molecule techniques as scanning electron microscopy (SEM) and cryo-transmission electron microscopy (cryo-TEM) as not to require the addition of chemical contrast agents or the fact to carry out the measurements at room temperature, respectively. AFM does not request the fluorescent labeling of the sample in contrast to other methods like stimulated emission depletion (STED) microscopy. The use of fluorescent dyes in biological research can lead toxicity on aquatic organisms (Rowiński and Chrzanowski 2010). Moreover, AFM enables data acquisition in liquid environments mimicking the near-physiological conditions inner the living cells, viruses or bacteria. The only need of AFM measurements is the properly biological sample fixation on a nanoflat solid surface in order to prevent the non-desirable dragging effects during the scanning runs. Table 1 established a comprehensive comparison of the main advantages and limitations displayed for the above described techniques. Here, the present scientific manuscript is focused on the use of AFM imaging, FS and nanoindentation operational mode techniques to elucidate the intrinsic properties of the microorganisms that live in aquatic ecosystems. Moreover, some recent examples are provided respect to other biological systems which could be fully extrapolated to those biodiversity involved in aquatic ecosystems.

AFM working principle

From its discovery in 1986, AFM has turned into one appealing technique to decipher multitude of properties. AFM consists of a flexible cantilever ended by an ultrasharp tip that scans a nanometrically flat surface where the sample of interest has been previously tethered by electrostatically or covalent immobilization strategies. Many methodologies have been developed on this regard (Valueva et al. 2020). Cantilevers are generally coated with reflective materials like gold or silver. A laser beam shines on the cantilever and trav-

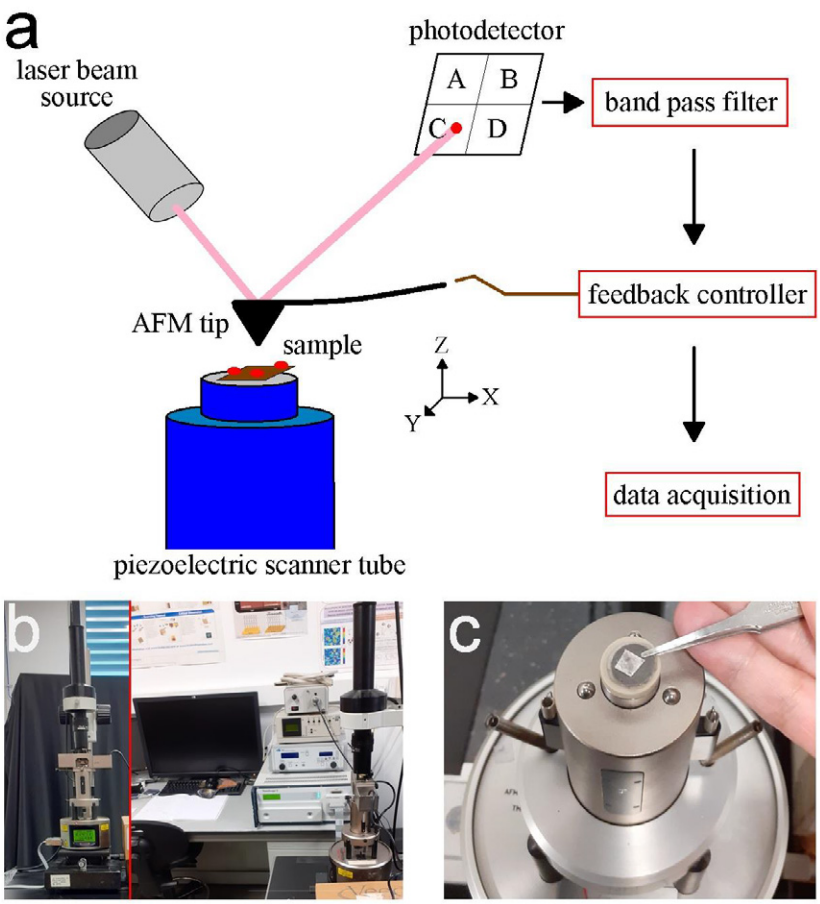

Fig. 1 (a) Schematic representation of an AFM setup. The AFM tip scans the sample surface while a laser beam is reflected towards a photodetector sensor by the AFM cantilever. The signal recorded by the photodetector is passed by a band filter. Then, the close-loop feedback controller provides the required excitation to the AFM probe to enhance the data acquisition quality. (b) Left hand: Commercial AFM multimode-8 (Bruker, Santa Barbara CA, USA). Right hand: Nanoscope V controller and PC computer. (c) Zenithal view of the sample loading on the piezoelectric scanner tube prior the data acquisition.

els to a photodetector device. The photodetector is divided into four quadrants. It exists basically three types of photodetector: I) A-B signal which is commonly used by the feedback loop of the AFM to monitor the cantilever deflection. This kind of photodetector is given by a position sensitive photodiode; II) Z-detector signal where the voltage is supplied by a strain gauge sensor which allows to monitor the vertical cantilever motion, and III) Y-stage signal that allows to monitor the lateral motion of the AFM stage. Then, the signal passes through a close-feedback loop maintaining constant one parameter depending on the mode of scanning. This fact keeps the probe-sample distance at a defined value. The sample is mounted on an iron steel disk by double side tape which is coupled to a piezoelectric scanner. Piezoelectric actuators are ceramic materials commonly fabricated from lead zirconium titanate which exhibits the capability to modify their dimensions in response to external applied voltages. The piezoelectric tube is vertically retracted or extended depending on the voltages furnished by the z-electrode. Whereas, piezo-tube is bent in the X-Y directions when voltage is applied to the respective electrodes. Finally, external sensors are employed to conduct attenuate corrections of the scanner height by Hardware operation. The typical lateral scan sizes achieved by piezoelectric scanners are 10 µm and 100 µm for biomolecules/living cells and bigger samples, respectively. The vertical range is 5-10 µm with sub-nanometer resolution which is fully applicable to study biological systems. The main drawback of piezoelectric scanners is the potential nonlinearity effects. This detrimental limitation is based on distortions like gain, offset and skew errors which are eliminated by applying image field corrections. The last AFM component is the electronics. The controller displays a real integrated lock-in based alternating current (AC) detection and demodulator to improve the measurement capability. Proportional-integral-derivative (PID) controllers deliver a prompt feedback according the signal acquired by the AFM setup. Fig. 1a depicts the main components of the AFM setup. Fig. 1b shows a real

AFM coupled with the respective controller device, whereas Fig. 1c displays how to handle the sample of interest before the AFM measurements. Clean tweezers with ultrapure isopropanol are needed to grab the iron disk where the sample is attached on the nanoflat solid surface (typically freshly cleaved mica). Mica is recommended for AFM experiments due to the facility to exfoliate it on surfaces with sub-nanometer roughness and also based on its low price. Furthermore, mica exhibits external hydroxyl groups (-OH) which enable to perform multitude of chemical procedures to tether the sample of interest on surfaces of this nature.

AFM imaging

AFM can operate in different image modes like contact, tapping and non-contact (C-AFM, TMAFM and NC-AFM). AFM tip taps the sample surface in C-AFM being the applied force the defined setpoint. TMAFM makes intermittent contact between the AFM tip and the external sample surface by controlling the resonance frequency of the cantilever. Finally, NC-AFM operates at extremely low cantilever oscillation amplitudes which enable to place the AFM tip in close contact position respect to the sample surface. The most recommended imaging operational modes for biological samples are TMAFM and NC-AFM in order to prevent the dragging of soft materials during scanning caused by the lateral forces. Here, some examples are provided to show the prospective capabilities of AFM imaging for the biological systems involved in aquatic ecosystems. I) Recently, AFM imaging has successfully monitored the structural changes of microcystin associations upon the presence of certain ionic metals in the media. Microcystins (MC) are heptapeptides produced by freshwater cyanobacteria under certain conditions. MC showcases human hepatotoxicity through contaminated water ingestion. AFM imaging revealed that MC binds Fe (III) ions which induce the growth of the MC strains (Ceballos-Laita et al. 2018). This finding makes toxic cyanobacteria more resilient by providing enhanced iron bioavailability. II) AFM imaging has been reported to visualize the interaction between ferric uptake regulator (Fur) proteins and DNA strands from cyanobacterium *Anabaena sp. PCCC 7120* (Pallarés et al. 2014). *Anabaena* is a filamentous, freshwater cyanobacterium capable of differentiating heterocyst cells for nitrogen fixation. AFM imaging resolved the underlying mechanism of sequential FurA association to DNA strands. This observation corroborates the regulation that FurA plays when DNA undergoes bending which results in a fine regulation in the Fur cluster associated genes. III) DNA repair complexes between Mre11 and Rad50 proteins from thermophilic archaeon *Sulfolobus acidocaldarius*. This aquatic microorganism could be a key player in future biotechnological applications (Quehenberger et al. 2017). AFM imaging provides topography maps to decipher the Mre11/Rad50 complexes taken place when it exists the unwinding of the DNA duplexes from *Sulfolobus acidocaldarius* (Zabolotnaya et al. 2020). IV) Microalgae cell morphology can also be addressed by AFM imaging (Demir-Yilmaz et al. 2021). Microalgae treasures fundamental interest rooted in their capability to convert water, inorganic nutrients and light into sustainable carbon-based value-added products. Changes on *Phaeodactylumtricornutum microalgae* cellular topography in presence of cadmium metal is a proof of the high-resolution delivered by AFM imaging (Ma et al. 2021).

AFM force spectroscopy (AFM-FS)

Nowadays, it exists multiple approaches to measure biological interactions like fluorescence cross-correlation spectroscopy (FCCS) (Slaughter et al. 2007), förster resonance energy transfer (FRET) (Rainay and Patterson 2019) or bioluminescence resonance energy transfer (BRET) (Pfleger et al. 2006), among others. Compared to the above described techniques, AFM force spectroscopy (FS) exhibits the main advantage to assess the adhesion properties of the sample of interest at the single molecule level. For example, the adhesion properties of rich β-sheet quaternary amyloid assemblies from *Paracentrotus lividus* sea urchin have been determined by FS revealing the increase cohesiveness in air conditions (Viana and Santos 2018). Then, the formation/dissociation of bonds involved in biomolecular complexes are influenced by the loading rates of the AFM lever. The force spectrum renders the prominent energy barriers traversed along the force-driven pathway by dynamic force spectroscopy (DFS). The dissociation parameters of the studied aquatic biosystems can be obtained by the Ritchie-Evans equation (eqn.1) (Evans and Ritchie 1997):

$$F^* = \left(\frac{k_B \cdot T}{x_\beta}\right) + ln\left(\frac{R \cdot x_\beta}{k_{off} \cdot k_B \cdot T}\right) \quad \text{(eqn.1)}$$

Where, the k_{off} and x_β are the dissociation rate at zero force and the reaction coordinate related to the spacing between the transition state projected and the involved chemical bonds, respectively. R is the loading rate, k_B is the Boltzmann constant and T is the temperature of the assayed measurement. The specific intermolecular interactions and the dissociation parameters of ferredoxin NADP$^+$ reductase (FNR) with its redox partners ferredoxin (Fd) and its substitute under iron-deficient conditions, flavodoxin (Fld) from cyanobacterium *Anabaena* PCC 7119 were assessed by DFS (Marcuello et al. 2015). DFS evidenced more specific interactions between FNR and Fd protein molecules being three-fold stronger rather than the complex formed between FNR and Fld. Moreover, the dissociation energy landscape of the complex established between FNR and Fd is through one single barrier whereas, FNR:Fld dissociates following two different energetic states. Recently, it has been found the dissociation parameters of the complex formed between wild type-FNR and relevant mutants with nicotin adenin dinucleotide phosphate (NADP$^+$) (Pérez-Domínguez et al. 2022). This study is the first time that the interaction between a flavoenzyme and its cofactor is addressed by DFS. The unbinding force driven by FNR:NADP$^+$ complexes yields nearby three- and seven-fold times greater than FNR:Fd and FNR:Fld, respectively. The k_{off} achieved for FNR:NADP$^+$ complexes is two magnitude orders smaller than the obtained for the flavoenzyme complexes from aquatic cyanobacterium *Anabaena* PCC 7119 which indicate the longer lifetime (τ) of FNR:NADP$^+$ complexes. Finally, the energy barrier heights (ΔG_β) from biological rupture forces can be obtained through eqn.2.

$$\Delta G_{\beta,0} = -k_B T * \ln(\tau_D * k_{off}) \quad \text{(eqn.2)}$$

When external forces are applied to the complexes, the energy barrier becomes to eqn.3.

$$\Delta G_\beta = \Delta G_{\beta,0} - x_\beta * F \quad \text{(eqn.3)}$$

This approach can be also applied for those biomolecules related to aquatic microorganisms as previously reported (Tapia-Rojo et al. 2017). DFS has been also devoted to study the interactions and the dissociation parameters of the complexes formed between non-aquatic organisms like the living cell membrane epidermal growth factor receptors (EGFR) and their respective ligand (EGF) molecules (Liu et al. 2019) or only the adhesion established between G protein-coupled receptors (GPCR) from Chinese hamster ovary (CHO) mammalian cells and anti-human influenza hemagglutinin (HA) antibodies (Dague et al. 2022), respectively. Table 2 summarizes the above described biological system examples tackled by DFS.

Unfortunately, no work related to aqueous living microorganisms has been published in this field. This aspect makes evident the necessity to develop new research lines in this area which will serve to better understand the gene expression of these aquatic microorganisms and how it is affected by cellular interactions (Armingol et al. 2021).

Nanoindentation

AFM allows the determination of rigidity parameters working the AFM tip as nanoindenter. AFM setup defines an indentation depth and the AFM tip penetrates the sample surface driven an elastic deformation. The Young's modulus (YM) is related to the sample stiffness (Butt et al. 2005) (eqn. 4 and eqn. 5):

$$k_s = 3/2 * aE^* \quad \text{(eqn.4)}$$

where k_s is the surface-sample stiffness, a is the AFM tip-sample contact radius and E^* is the reduced YM.

$$\frac{1}{E^*} = \frac{3}{4}\left(\frac{1-v_s^2}{E_s} + \frac{1-v_t^2}{E_t}\right) \quad \text{(eqn.5)}$$

v_t and v_s are the ratio transverse strain to axial strain of the tip and the surface, respectively (also known as Poisson's ratio). The parameters E_s and E_t are the YM of the sample and the tip, respectively.

Table 2 Compilation of the mean unbinding force values at R = 10 nN/s for FNR:Fd, FNR:Fld and FNR:NADP$^+$ complexes and R = 1 nN/s for EGFR:EFR complex, respectively. k_{off} and x_β dissociation parameters are also depicted in combination with the number of observed transitions during the dissociation process.

Biological complex	Mean rupture force (pN)	k_{off} (s^{-1})	x_β (nm)	Number trends (Ref)
FNR:Fd (*Anabaena* PCC 7119)	57	21.2	0.27	One (Marcuello et al. 2015)
FNR:Fld (*Anabaena* PCC 7119)	21	5.7	0.47	Two (Marcuello et al. 2015)
FNR:NADP$^+$ (*Anabaena* PCC 7119)	136	2.0 10^{-2}	2.1 10^{-2}	One (Pérez-Domínguez et al. 2020)
EGFR:EGF (living cell)	100	1.2	0.13	Two (Liu et al. 2019)

Several theoretical models have been developed to estimate the YM gathered from experimental data. The most used are the following: I) the Hertz (Hertz 1882), II) Johnson-Kendall-Roberts (JKR) (Johnson et al. 1971), and III) Derjaguin-Muller-Toporov (Derjaguin et al. 1975) models. Hertz model takes into account just fully elastic contact, whereas JKR and DMT models consider the fully elastic contact with adhesion forces inside the contact zone and these adhesion forces in combination with Van der Waals interactions around the contact area, respectively.

Nanoindentation experiments have been deeply exploited to decipher the YM of multitude of organisms living in aquatic ecosystems. First, the adhesive mucilage layers released by benthic diatoms of *Crasepdostauros australis* and *Pinnularia viridis* marine species showed different rigidity parameters (Higgins et al. 2003). The YM of *C.australis* and *P.viridis* is 0.45 ± 0.01 MPa and 0.76 ± 0.03 MPa, respectively. These findings can aid to create materials with unprecedented adhesive performance. Secondly, nanomechanical properties of algal cells like the marine green flagellate *Dunaliella tertiolecta* (Pillet et al. 2019). The most relevant outcome of this work is the loss of elasticity between the exponential and stationary phases (YM ranges from 31.0 ± 4.7 kPa to 19.0 ± 7.3 kPa, respectively). This decay of nearby 40 % is an effect to the inherent cellular aging processes. Other illustrative example is the nanoindentation study of brown algal cells (Tesson and Charrier 2014). Authors found a large variability of YM values (1-100 MPa) being three magnitude orders softer compared to plant polymers (Gerbin et al. 2020). This observation can be due to the adaptability behavior of algal microoganisms to environmental changes. In this framework, algal organisms have developed buoyancy response upon mechanical stress through the growth of pneumatocyst cells (Stewart 2006). These cellular variations confer plasticity to algae populations. Then, the YM of the membrane Sfp1 adhesive proteins from sea stars has been also interrogated by nanoindentation (Lefevre et al. 2021). The YM varies from 0.5 ± 0.1 MPa to 500 ± 100 MPa when the nature of Spf1 proteins expressed by recombinant sea stars and salt concentration in the medium are modified. The results evidence the strong impact of environmental conditions on nanomechanical properties of the studied organisms. Finally, the elastic modulus of *Prorocentrum donghaiense* which is a class of planktonic dinoflagellate species has been also addressed under nitrogen limitation (He et al. 2022). Nanoindentation measurements revealed the increase of the nominal YM from 0.6 MPa (normal culture conditions) to 3.4 MPa (in absence of nitrogen). This aspect suggests the strong capability of cells to change their transcriptional expression which is regulated by mechanical stress. Table 3 depicts the aforementioned YM values obtained by nanoindentation measurements for organisms coming from aquatic ecosystems.

The considerable variability of the gathered YM values for the organisms studied from aquatic ecosystems is rooted in their adaptability to the environment. This fact promotes the survival of these species.

Conclusions and future perspectives

Aquatic ecosystems treasure a high richness in terms of flora and fauna and not always are evident their intrinsic properties. In this framework, AFM has demonstrated to gather multitude of physico-chemical properties with extreme accuracy (Marcuello 2022). Since AFM enables to acquire data in liquid media mimicking the intracellular conditions, reliable information can be obtained being transposed to the living

Table 3 Young's modulus values of the studied aquatic microorganisms under relevant conditions.

Biological complex	Mean Young's modulus	(Ref)
Benthic diatoms (*P. viridis*)	0.76 ± 0.03 MPa	(Higgings et al. 2003)
Benthic diatoms (*C. australis*)	0.45 ± 0.01 MPa	(Higgins et al. 2003)
Algal cells (*Dunaliella tertiolecta*)	31.0 ± 4.7 kPa (exponential phase)	(Pillet et al. 2019)
Algal cells (*Dunaliella tertiolecta*)	19.0 ± 7.3 kPa (stationary phase)	(Pillet et al. 2019)
Brown algal cells	1-100 MPa	(Tesson and Charrier 2014)
Sfp1 Delta 150 mM $CaCl_2$ (Sea star)	0.5 ± 0.1 MPa	(Lefevre et al. 2021)
Sfp1 Beta C-term 0.45 M NaCl (Sea star)	500 ± 100 MPa	(Lefevre et al. 2021)
Plankton (*Prorocentrum donghaiense*)	0.6 MPa (normal conditions)	(He et al. 2022)
Plankton (*Prorocentrum donghaiense*)	3.4 MPa (absence of Nitrogen)	(He et al. 2022)

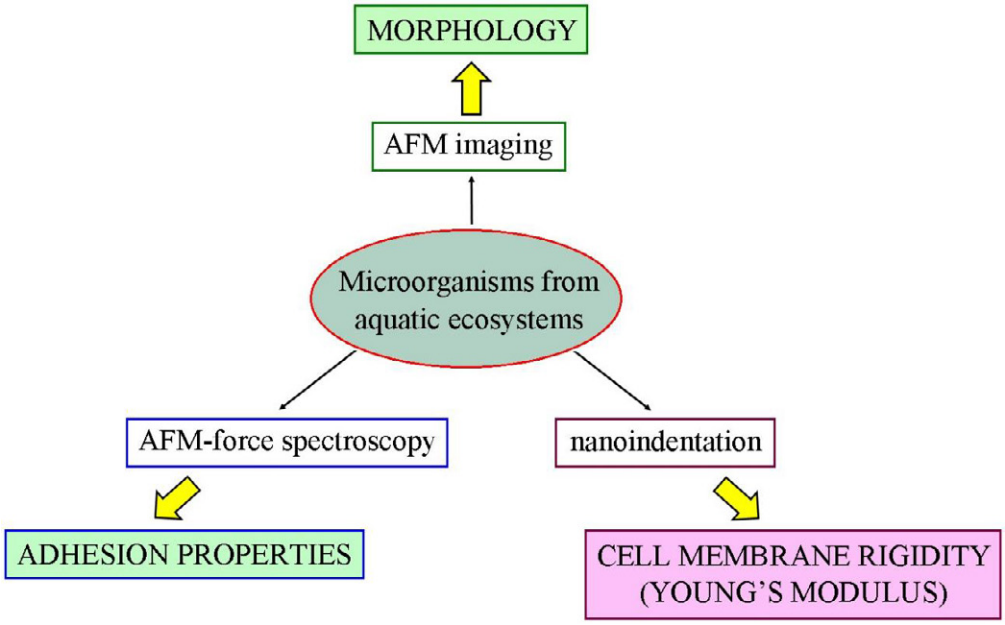

Fig. 2 Schematic representation of the applications driven by AFM imaging, AFM-force spectroscopy and nanoindentation to get insights from microorganisms living in aquatic ecosystems.

aquatic ecosystems. The present work is focused on AFM imaging, AFM-force spectroscopy and nanoindentation measurements which serve to assess the morphology, adhesion and rigidity properties (Fig. 2).

It exists promising future perspectives in this field. Recently, the development of methodologies to analyze the AFM imaging raw data through volumetric statistical analysis makes possible to discern morphological changes upon ligand and catalysis (Marcuello et al. 2021). Moreover, the design of functionalization AFM tip procedures to covalently attach biomolecules of interest oriented towards its respective partners combined with AFM-force spectroscopy adhesion maps render quantitative molecular recognition imaging (Marcuello et al. 2022). Molecular recognition can be fully extensible to identify biomolecular and/or cellular hybrid samples coming from aquatic ecosystems. This approach can be applied to devise ultrasensitive detection technologies or the creation of high-throughput adhesive bio-inspired materials for industrial purposes as binders. Finally, nanoindentation not only can be exploited to interrogate biology aquatic systems but also, all non-biological sources eventually present in these ecosystems. One outstanding example is the recent study to determine the rigidity and distribution of microplastics inside human fibroblastic cells (Akhatova et al. 2022). This work can be also devoted in marine species to monitor the microplastic uptake along the emplacement. In future, cell mechanics can be act as an environmental fingerprint marker to visualize the adaptation and viability of marine populations in aquatic ecosystems. Finally, the analysis

of the antioxidant response of some organisms like microalgae (Ugya 2021) or the impact of stocking density of plankton communities (da Silva et al. 2022) on the phycoremediation of wastewater coming from human industrial activities can pave the way to design the next-generation of methodologies to protect the water bodies and the use of highly promising nanotechnological tools like AFM can act as one of the main cornerstones to successfully reach these purposes. Some solutions have been offered like the use of floating treatment wetlands (FTWs) combined with pollutant-degrading bacteria (Do et al. 2021), but more research must be carried out in this field.

Funding This research received no external funding. The author acknowledges the funding that covers his salary coming from EU Recovery action 2021 (CSIC-project code: QTP2103003).

Data availability statement Additional data is available upon reasonable request.

Acknowledgments CM would like to acknowledge the use of Servicio General de Apoyo a la Investigación-SAI, Universidad de Zaragoza. CM acknowledges the use of instrumentation as well as the technical advice provided by the National Facility ELECMI ICTS, node "Laboratorio de Microscopias Avanzadas" at Universidad de Zaragoza.

Conflicts of interest The author declares no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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