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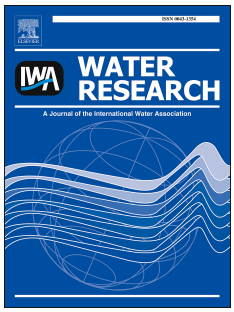

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The use of diatoms in ecotoxicology and bioassessment: insights, advances and challenges

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Abstract

Diatoms are regularly used for bioassessment and ecotoxicological studies in relation to environmental and anthropogenic disturbances. Traditional taxonomical diatom parameters (cell counts, biovolume estimates, species richness, diversity indices and metrics using sensitive and tolerant diatom species) are regularly used for these studies. In the same context, very less focus was given on new endpoints of diatoms (life-forms, nuclear anomalies, alteration in photosynthetic apparatus shape, motility, lipid bodies, size reduction and deformities), in spite of their numerous merits, such as, their easiness, quickness, cheapness, global acceptation and no especial training in diatom taxonomy. In this review we analyzed 205 articles (from lab and field studies), with the aim to investigate the bioassessment and ecotoxicological advancement taken place in diatom research especially in terms of exploring new endpoints along with the traditional taxonomical parameters in a perspective which can greatly enhance the evaluation of fluvial ecosystem quality for biomonitoring practices.

Keywords- Biomonitoring; Ecotoxicology; Nuclear anomalies; Lipid bodies; Size reduction; Deformities

1. Introduction

Globally, diatoms are used to assess the ecological status of aquatic systems because diatoms have a cosmopolitan nature, short life span and quick response to environmental and anthropogenic disturbances (Stevenson et al., 2010). Such use of diatoms is meaningful, given that diatoms are the chief primary producers in waterbodies, contributing 40 % of the primary productivity of the oceans and contributing approximately 20 % of global carbon fixation (Hildebrand, 2008). Various organisms (macroinvertebrates, fishes, macrophytes and algae, including diatoms) have been used for biomonitoring around the world, but among these organisms, diatoms are the most suitable for assessing the chemical status of waterbodies (McCormick and Cairns, 1994; Stevenson et al., 2010). For example, Hering et al. (2006) found that diatoms were sensitive to nutrient and organic matter contamination, whereas fishes, macroinvertebrates and macrophytes were more sensitive to hydrological changes in aquatic ecosystems. Indeed, the high sensitivity of diatoms to organic toxicants (atrazine, metolachlor, simazine, phenols and PAHs) (Blanco and Bécares, 2010), organic matter (especially nutrients, nitrate and phosphate) (Stevenson et al., 2010; Stevenson, 2014; Morin et al., 2016) and inorganic contamination (heavy metals) (Hirst et al., 2002; De Jonge et al., 2008; Morin et al., 2012) has been reported from different parts of the world. A major advantage of using diatoms in environmental studies, including ecotoxicology, is that diatom assemblages are specious and can be used to investigate the effects of toxicants at different levels of ecological organization (community, population, and

individual levels) (McCormick and Cairns, 1994; Debenest et al., 2013; Stevenson, 2014). Because of these characteristics, a variety of national and international agencies have recommended diatoms as a biomonitoring tool for assessing the ecological status of rivers and streams (Kelly et al., 1998; Stevenson et al., 2010).

Diatoms are characterized by their robust, ornamented, species-specific siliceous frustules, which are preserved and dependably replicated in successive generations (Falasco et al., 2009). Species identification is based on these siliceous frustules. Even in fossilized form, diatom frustules are a useful tool to assess the paleoclimatic conditions (Mackay, 2007). Although used for biomonitoring worldwide, few studies have related diatom diversity in Polar Regions to global climate change (Anderson, 2000; Bopp et al., 2005; Alvain et al., 2013).

In spite of their diversity, beauty, ecological importance and biomonitoring potential, diatoms are globally underutilized as a tool for risk assessment and for evaluating management options for fluvial ecosystems (Stevenson et al., 2010). The main reason behind this underutilization is the limited types of metrics traditionally used in bioassessment. Traditional metrics for diatoms include biovolume, cell density and relative abundance (with special reference to indicator species), whereas the newer behavioral, physiological and functional metrics are rarely used. These newer metrics have advantages in understanding the dynamics of biological communities (Giddings et al., 2002), especially because these metrics demonstrate sublethal effects that are not apparent in diatom counts (the basis of traditional metrics).

Community structural metrics are useful for predicting possible adverse effects of chemicals at the population and community level, thus they are directly linked to

biodiversity. According Knauer and Hommen (2012), two common metrics - species richness and Shannon-Wiener index (H') - showed low bioassessment value for community structure, whereas total abundance and the abundance of the dominant species demonstrated high sensitivity.

High diatom community variability is an inherent characteristic of complex test systems, such as in experimental micro- and mesocosm studies, and consequently, this variability complicates interpretation of studies (Campbell et al., 1999). Therefore, evaluation of test systems is preferably not based on biodiversity metrics alone, but is complemented by other parameters (e.g., functional metrics) that are quick, easy and can be used globally. Functional metrics are pertinent because they are closely linked to some regulatory and supporting ecosystem services, and may be less variable among replicates. Furthermore, even if the structural integrity of the community is altered, some functional parameters (such as primary productivity and nutrient cycling) may be unaffected and vice versa. Combining information from both structural and functional metrics enhances the sensitivity and predictive power of studies aimed at bioassessment and risk assessment. Indeed, the use of both structural and functional parameters provides sensitive and powerful early warning tools for evaluating sub-lethal effects of exposure to toxins (Renzi et al., 2014). However, structural metrics (cell counts and biovolume, species composition and abundance) had high variation due to variation among replicates, which resulted in statistically weak relationships with the studied stressors (Kraufvelin, 1998; Knauer et al., 2005). Varying division rates of individual diatom species (which is often related to their size; Lavoie et al., 2006) is a prime reason for high variation among test replicates, in part because accurate quantification of diatom frustules is still a major

104 constrain for ecotoxicological studies. Thus, there is an urgent need to develop or
105 incorporate more sensitive metrics for assessing early exposure to stress, specifically
106 metrics that measure physiological or morphological changes in diatom species, making
107 functional metrics a priority for further research (Renzi et al., 2014).

108 For bioassessment and ecotoxicological practices several new diatom parameters
109 have been reported in the last decade; parameters that are very promising but sporadically
110 used or reported. These parameters are nuclear anomalies (Debenest et al., 2008; Licursi
111 and Gómez, 2013), alteration in the cell membrane, cytoplasmic content and
112 photosynthetic apparatus (Chang et al., 2011; Armbrecht et al., 2014; Wood et al., 2014),
113 changes in lipid body formation (Pandey et al., 2015) and the classification of diatoms
114 using various life-forms and ecological guilds (Passy et al., 2007; Rimet and Bouchez,
115 2011). Relationships among these unconventional parameters have been found in some
116 studies. For example, diatom motility was associated with the size/number of lipid bodies
117 (Wang et al., 2013), size reduction can be associated with frustule deformity (Hasle and
118 Syvertsen, 1996), lipid bodies metrics and frustule deformities may vary with
119 cytoplasmic anomalies (Renzi et al., 2014) and frustule deformity is associated with lipid
120 body characteristics (Pandey et al., 2015). This correlation among unconventional metrics
121 demonstrates shared sensitivity and indicates their efficiency in measuring responses to
122 toxicants, especially early effects and cellular responses to high toxicant doses. These
123 metrics may allow early detection of stress after exposure to doses below those needed to
124 cause cell death, which is the endpoint detected by traditional methods (Renzi et al.,
125 2014). In addition, these unconventional parameters are also reported to be effective for
126 bioassessment practices. For example, Gillet et al. (2009, 2011) reported percent live

diatoms in the biofilm to be a cost-effective bioassessment tool. Similarly, Morin et al. (2016) advocated the use of impaired cytoplasmic content in live diatoms for the bioassessment of fresh waterbodies. Furthermore, various life-forms and ecological guilds of diatoms are regularly investigated for assessing the ecological health of waterbodies (Medley and Clements, 1998; Passy, 2007; B-Béres et al., 2014). These newer metrics are quick, easy, require less human expertise, have good reproducibility, have standard protocols and, most importantly, can be adopted world-wide. However, the use of these metrics for ecotoxicological and bioassessment testing needs further study and demonstration in order to better determine concentration-dependent and/or time-dependent responses and to gain wider acceptance (Dickman, 1998; Renzi et al., 2014).

The use of molecular approaches (next-generation sequencing, DNA barcode, DNA fingerprinting) in diatom-based biomonitoring practices has recently received significant attention (Kermarrec et al., 2013; Kermarrec et al., 2014; Manoylov, 2014; Visco et al., 2015). Developments of next-generation sequencing technologies offer the possibility to use molecular barcoding for fast and reliable biodiversity surveys based on environmental samples. Next-generation sequencing approaches have tremendous potential for diatom-based monitoring, such as ease in species identification, comparison of species inventories, real-time assessment of living communities and reduction in sample processing time (Visco et al., 2015). Similarly, DNA fingerprinting may be used to measure the genetic diversity in populations of diatoms (e.g., *Ditylum brightwellii*) and may also help understand the relationship between diatom blooms and environmental conditions (Rynearson and Armbrust, 2000). Molecular bioassessment of diatoms is a promising field but at this point, we cannot yet adopt them for many reasons. The identity

of most taxa in genetic reference libraries has not been rigorously evaluated, so reference library based taxonomy may not be accurate (Manoylov, 2014). Lastly, the high cost of reagents per megabase sequencing output and the reading of homopolymer regions are additional issues with molecular bioassessment that need to be addressed (Claesson et al. 2010). As a consequence, the number of field-collected samples analyzed for bioassessment with molecular tools has been limited (Kermarrec et al. 2013).

Incorporating the new metrics along with the traditionally used metrics will make diatom ecotoxicological and bioassessment practices easier and more rapid, reproducible, cheap and globally accepted. Thus, in this review, we explore the present status of various structural and functional metrics of diatoms used in ecotoxicology and bioassessment studies and discuss their merits and demerits in order to raise the profile of diatoms as an effective tool for biomonitoring practices.

2. Traditional endpoints

2.1. Diatom diversity and abundances

Analysis of available literature does not support a direct cause-effect relationship between chemical pollution and diatom diversity. In general, the relationship between diversity and chemical contamination is not always a simple linear positive/negative association and results are not always strong enough to indicate a management approach that would most effectively conserve the health of the ecosystem (Ricciardi et al., 2009). Thus, a careful analysis of how loss of diversity may be linked to chemical contamination would be useful in refining risk-assessment procedures. Effects of contaminants on diatom communities have been frequently evaluated using various diversity indices, such as species richness, Shannon index, evenness, and Jaccard similarity index. For example,

Gold et al. (2002) used a translocation experiment and reported lower cell density, species richness and Shannon index of the periphytic diatom community at the severely polluted sites. Similarly, Verb and Vis (2005) reported lower diversity (in terms of species richness and Shannon index) of periphytic diatoms at the acid mine drainage (AMD) impacted sites in the USA. Recently, Morin et al. (2015) found significantly higher values for various diversity indices for periphytic diatoms (species richness and Shannon index) from the control sites (Firmi and Moulin) than from contaminated sites (Joanis and Usine for metals and Decazeville for high nutrient loads) of the Riou-Mort River, South West France. Similarly, Luís et al. (2011) reported lower Shannon index values of periphytic diatom communities examined at acid mine drainage sites in Portugal. Moreover, reproduction of diatom species may be inhibited under stress, resulting in decreases in species richness and diversity, as has often been reported at metal polluted sites (Deniseger et al., 1986; Genter and Lehman, 2000).

These biodiversity parameters are regularly used but sometimes do not provide consistent information about the known impairment of waterbodies (Blanco et al., 2012). For example, Hirst et al. (2002) showed that species richness, evenness and diversity were not significantly related to the chemical characteristics of stream water polluted by mining activities in United Kingdom. In the same context, De Jonge et al. (2008) reported statistically insignificant correlations between periphytic diatom community parameters (diversity, evenness and different indices) and the heavy metal load of water and sediment in a lowland river in Flanders, Belgium. Similarly, Duong et al. (2008) found no difference in the diversity indices of periphytic diatom communities examined at the reference (Firmi) sites and contaminated (Joanis) sites of the Riou-Mort River,

196 South West France. Duong et al. (2010) also found no significant difference in terms of
197 species richness (S) and diversity (H′) between the control periphytic diatom community
198 and that contaminated with 100 µg L^{-1} Cd after one week of colonisation. However,
199 significantly higher metric values were recorded in the control community than in the
200 contaminated community at the fourth and sixth week of the experiment.
201 Other studies showed mixed responses of diatoms against different types of
202 stresses. For example, Medley and Clements (1998) reported significantly lower species
203 diversity ($p < 0.05$) but the relation was insignificant in terms of cell density, species
204 richness and abundance of dominant taxa at the Zn polluted sites in the Colorado Rocky
205 Mountain streams (USA), whereas Hill et al. (2000) were unable to relate species
206 richness with metal concentration in a Rocky Mountain stream polluted with heavy
207 metals. Gold et al. (2003a) reported higher Shannon index (H'), the same species richness
208 and a low cell density at the metal polluted (Cd and Zn) sites in comparison to reference
209 sites. In contrast, Sabater (2000) found that heavy metals in water and sediments were
210 significantly and negatively affecting various diatom metrics (evenness, diversity and
211 diatom indices). In addition, they also reported marked and long-lasting effects of heavy
212 metals on the periphytic diatom communities from impacted versus reference sites of the
213 Guadiamar River, South-West Spain. This inconsistency of diatom diversity indices and
214 lack of significant correlations between diversity, evenness and environmental variables
215 is in accordance with the "Intermediate Disturbance Hypothesis", which assumes a
216 parabolic relation between diversity and water quality, with the highest diatom diversity
217 at intermediate pollution levels (Van de Vijver and Beyens, 1998; Hirst et al., 2002).

Ricciardi et al. (2009) reviewed the status of biological communities in rivers in relation to chemical contamination and reported two major constrains in diversity and pollution studies. First, they found that the use of a single diversity index or small number of diversity indices was inadequate. The different biodiversity indices assess different types of information and using only one of these indices (types of information) is not enough to adequately define biological diversity. Multivariate-analysis studies that combine both conventional and taxonomy-based indices and possibly different trophic levels provide a more comprehensive view of the diversity status of an ecosystem and its potential response to pollutants. Second, they advocated the use of coupled chemical and biological analyses to evaluate water-quality status and the effect of chemicals on fluvial biological diversity, thus promoting a multidisciplinary approach. Further, they recommended incorporating a variety of types of biodiversity indices, especially when using algae (diatoms) for assessing the chemical status of aquatic systems. According to Stevenson et al. (2010), composite indices that incorporate both the richness and evenness elements of diversity are needed to characterize the kind and severity of pollution in fluvial ecosystems. Similarly, Blanco and Bécares (2010) found that the simultaneous use of several diatom indices is required to detect and evaluate the huge spectrum of potential pollutants in river basins.

Changes in species composition tend to be the most sensitive response when comparing impacted and reference diatom assemblages (Stevenson, 1984; Jüttner et al., 1996). Species composition is often examined as relative abundance of diatom species in the community; a metric frequently used for deciphering the ecological health of waterbodies (Medley and Clements, 1998; Gold et al., 2002, 2003a; Morin et al., 2008a;

241 De Jonge et al., 2008; Stevenson et al., 2010; Duong et al., 2012; Morin et al., 2012;
242 Arini et al., 2012a,b,c; Pandey et al., 2014, 2015, 2016; Gautam et al., 2017). Lavoie et
243 al. (2006) reported relative abundance as a more informative tool than other community
244 parameters (especially biovolume of the community) for biomonitoring. In this study,
245 analyses conducted on diatoms grouped by size showed that small and large taxa have
246 similar responses to the water chemistry variables (total phosphorus, soluble phosphorus,
247 total dissolved nitrogen, nitrates, ammonia, temperature, dissolved oxygen, conductivity
248 and dissolved organic carbon). Furthermore, they found that biovolume estimation
249 analysis provided no extra information but required considerably more time, money and
250 human expertise. Similarly, Litchman et al. (2008) concluded that data on the relative
251 abundance of different diatom sizes in diverse aquatic ecosystems may indicate high
252 variation in distribution, reflecting a variety of immediate, system-specific selective
253 pressures. Stevenson et al. (2010) also found relative abundance of diatom genera and
254 species as the most valuable attribute of diatom assemblages because several multimetric
255 indices of biotic and ecological condition have been developed for bioassessment using
256 relative abundance. Furthermore, Schindler (1990) reported that in most field sampling
257 situations, species composition should be more sensitive to changes in environmental
258 conditions than changes in biomass or metabolic rates, especially when stresses have
259 existed long enough for immigration of new species and accrual of rare taxa that are
260 stress-tolerant.

261 Relative abundance data can become informative through the use of ordination,
262 clustering and similarity indices (Stevenson et al., 2010). More specifically, ordination-
263 based multivariate analysis (e.g., correspondence analysis, detrended correspondence

analysis, non-metric multidimensional scaling, principal component analysis and distance-based redundancy analysis) was developed to relate community composition to measured variation in the environment. Ordination plots obtained in the analyses are linear combinations of environmental variables, along which the relative distributions of species assemblages. Ordination plots help in visualizing the pattern of community variation, distributions of species and assemblages among environmental variations, and can be used for indicating species-environment relationships. (ter Braak, 1986; ter Braak, 1987; ter Braak and Šmilauer, 2002). Furthermore, ordination and clustering can be used to show assemblages that differ from other assemblages, which may be caused by anthropogenic impacts (e.g. Chessman, 1986; Stevenson and White, 1995). Thus, incorporation of these analyses (any one) provides a simple, easy-to-understand description of the percent change in species relative abundances between assemblages at assessed and reference sites.

Diatom diversity indices and their relative abundance in the diatom communities are widely used to indicate the presence and extent of organic and inorganic contaminants in fluvial ecosystems. In most studies, these routinely used indices provide a statistically weak relationship between diatom diversity and pollutants. Thus, in the light of this result, there is an urgent need to incorporate new types of diatom-based indices (e.g., a taxonomic composition based index), which can provide reliable information using statistical tools. Improvement in taxonomic resolution of diatom species, use of composite indices (richness, evenness, diversity and dominance), increased cell counting (3000-8000 valves) and simultaneous use of several indices to detect and evaluate the huge spectrum of potential pollutants have been proposed to improve the efficacy of

287 diatom-based ecotoxicological assessments (Blanco and Bécares, 2010; Stevenson et al.,
288 2010; Rimet and Bouchez, 2011).

289 *2.2. Cell densities and biovolume of the community*

290 Changes in community cell densities and biovolume represent an important tool for
291 diatom-based community bioassessment (Pandey et al., 2014). Diatom densities are
292 estimated using various methods, such as counting cells with a Spencer's
293 haemocytometer at 450x magnification, and densities are converted to biovolume by
294 applying geometrical volume formulas for generalized diatom shapes (Hillebrand et al.,
295 1999). Alteration in the cell density and biovolume of the diatom in biofilms has been
296 variously attributed to current velocity (Ghosh and Gaur, 1998), diatom emigration and
297 immigration (Stevenson and Peterson, 1991), grazers (Colletti et al., 1987; Arini et al.,
298 2012b), pesticides (Pérès et al., 1996; Debenest et., 2009; Debenest et al., 2010; Morin et
299 al., 2010) and heavy metals (Gold et al., 2002; Pandey et al., 2014). Cell density and
300 biovolume changes (either single or together) following the translocation of diatom
301 assemblages between non-impacted and impacted locations has been used to assess the
302 level of disturbance in the waterbodies (Gold et al., 2002, 2003a,b; Duong et al., 2012;
303 Arini et al., 2012a,b,c). Under heavy metal stress, translocation-based studies indicated
304 differential response for the direction of translocation. Specifically, in moving from
305 polluted to unpolluted conditions, total diatom density showed rapid changes (in 2
306 weeks), but this change was much slower in the opposite direction, in that the worsening
307 water quality imposed on the very dense biofilm matrix from non-polluted conditions
308 took more than 4 weeks to show clear modifications, despite the presence of

309 contaminants, the poor conditions of oxygenation, and high levels of nutrients (Gold et
310 al., 2002; Duong et al., 2012).

311 Although diatom densities in biofilms have been widely reported as a relevant
312 bioindicator of heavy metal contamination (Gold et al., 2003a,b; Morin et al., 2008a;
313 Duong et al., 2010; Morin et al., 2010), the usefulness as a bioassessment tool has not
314 been consistent and has been criticized. Major reasons for this criticism is not including
315 biotic variable (e.g., immigration, emigration, grazers etc.) (Stevenson and Peterson 1991;
316 Stevenson et al., 1991; Sabater et al., 2002; Arini et al., 2012b) and abiotic variables
317 (substrates, pH, conductivity, alkalinity) (Medley and Clements, 1998; Potapova and
318 Charles, 2005) that can also strongly influence diatom densities. For example, heavy
319 metal contamination can indirectly affect diatom cell density by reducing the density of
320 grazers in contaminated sites, which can lead to the unexpected finding in some field
321 studies that higher diatom cell densities occurred at contaminated sites than at reference
322 sites (Guasch et al., 2016). Sabater et al. (2002) suggested the need for closer attention to
323 grazing effects on biofilm function, since grazing can affect the performance of biofilms
324 in the amelioration of river water quality. Diatom cell density response to pesticide
325 exposure may not be consistent (Debenest et al., 2010). For instance, atrazine exposure
326 may decrease cell density in some centric diatom species, and increase cell density for
327 some pennate diatom species (Bérard and Benninghoff, 2001; Bérard et al., 2004). In
328 contrast, another herbicide, isoproturon, consistently impacts diatom cell densities (Pérès
329 et al., 1996; Schmitt-Jansen and Altenburger, 2005; Debenest et al., 2009).

330 Biovolume estimation of diatom communities has been used for bioassessment, as
331 an alternative to cell counts for representing the level of stress in diatom biofilms (Ricart

et al., 2009; Pandey et al., 2015). Cell biovolume has the advantage of measuring the contribution of each algal group or taxa relative to primary production (Hillebrand et al., 1999; Lavoie et al., 2004; Cloern and Dufford, 2005; Larras et al., 2014b). Cell biovolume may have another advantage. Under stress, diatom density may increase or be unchanged because of the disproportionate growth of small and adnate diatoms (Medley and Clements, 1998; Gold et al., 2002, 2003a,b; Duong et al., 2012; Arini et al., 2012b,c; Morin et al., 2008a; Morin et al., 2012; Leguay et al., 2016; Pandey and Bergey, 2016), which may lead to erroneous findings based on cell counts; that is, that the investigated system may not be under any type of stress. In this case, use of community biovolume would be more appropriate for bioassessment than cell counts. As an example, Arini et al. (2012b) reported a gradual increase in cell density with gradual decrease in diatom biovolume of the community at sites heavily contaminated with heavy metals in a laboratory experiment. Similarly, Barral-Fraga et al. (2016) reported appreciable reduction in mean (approximately halved) and total diatom biovolume in a diatom dominated biofilm exposed to arsenic versus the control, which had no apparent difference in biofilm cell density. Alternatively, no pattern in density and biovolume of the diatom community have been reported under stress (Arini et al., 2012b,c). Chemical exposure can produce similar results. Ricart et al. (2009) reported a concentration dependent decrease in the diatom biovolume under exposure to the herbicide diuron, which was associated with the predominance of smaller growth forms in the biofilm (Sabater and Admiraal, 2005). In addition, Ricart et al. (2009) found that the EC_{50} values for the biovolume were extremely low, indicating that this was a highly sensitive parameter to diuron exposure, and that effects might be significant in nature. Furthermore,

355 NEC and EC50 values obtained with the community biovolumes were generally lower
356 than the ones obtained using short-term single-species tests, as registered in the US EPA
357 database (i.e. Gatidou and Thomaidis 2007 (EC50 = 27 μg L^{-1}, *Navicula forcipata*,
358 growth); Ma et al., 2002 (EC50 = 4.3 μg L^{-1}, *Chlorella vulgaris*, growth); Podola and
359 Melkonian 2005 (EC50 = 6.4 μg L^{-1}, *Cryptomonas* sp., chlorophyll-a fluorescence). This
360 comparison emphasizes the relevance of using natural communities to test environmental
361 effects.

362 Traditionally used community parameters (e.g., cell density, biovolume) provide
363 useful information about the nature of community but there is a major drawback. These
364 parameters are investigated by counting 500-1000 diatom frustules in each sample. The
365 quality of this well-accepted practice has been criticised (Hillebrand et al., 1999;
366 Takabayashi et al., 2006). Accurate frustule counts require taxonomic expertise of
367 diatoms, a skill that is often hampered by inadequate or not readily assessable taxonomic
368 resources. Furthermore, small size of diatoms, even of the most common taxa, can be
369 difficult to identify using light microscopy and hence making the investigation more
370 complicated. Some diatom species or individuals may be in valve, girdle, or tilted
371 orientations when viewed and diatoms in different orientation look markedly different
372 from each other, making diatom identification more complicated. Additionally, counts
373 require a lot of time (and, hence, expense). More specifically, investigation of diatom
374 community biovolume is regularly criticized as it requires time-consuming measurements
375 of diatom frustule dimensions (length, width and height) followed by estimation of
376 biovolume, using formulae based on generalized diatom shape, which will again increase
377 the time and cost of research.

2.3. Sensitive and tolerant species

Diatoms inhabit all types of waterbodies and contain troves of diversity in terms of taxonomy, morphology and ecology (Larras et al., 2014a). Due their rapid turnover time, diatoms respond quickly and also recovery much faster than other algae (Cattaneo et al. 2004; Morin et al., 2008a; Stevenson et al., 2010). Different species of diatoms respond differently and characteristically to environmental (e.g., pH, salinity, ionic content, dissolve organic carbon; Sabater et al., 2007; Potapova and Charles, 2007) and anthropogenic (e.g., nutrients, heavy metals, pesticides, herbicides; Kelly and Wilson, 2004; Falasco et al., 2009; Larras et al., 2013) stressors (Fig. S1). Because of these characteristics, diatoms are used globally for assessing the ecological health of waterbodies.

Although individual diatom species may be good indicators of certain conditions, the genus level is often also useful. For example, at the genus level, certain diatoms (*Eunotia*, *Fragilaria*, *Navicula*, *Nitzschia* and *Pinnularia*) can be excellent bioindicators for acid mine drainage (AMD) (DeNicola, 2000; Morin et al., 2012; Pandey and Bergey, 2016). Similarly, Larras et al. (2014a) found diatom sensitivity to herbicides and reported that centric and araphid diatoms (Thalassiosirales and Fragilariales, respectively) included the most sensitive species whereas most resistant species were mainly pennates (Cymbellales, Naviculales and Bacillariales). Meanwhile, Van Dam et al. (1994) found that the genera *Nitzschia* and *Achnanthidium* were characteristic of polluted waters receiving either nutrients or organic biodegradable materials, *Pinnularia* and *Cymbella* were found proportionally more numerous in pristine conditions than polluted ones and the abundant genus *Navicula* was ambiguous in terms of the water quality.

In contrast, many authors suggest that species-level identification of diatoms is adequate for routine biomonitoring (Pérès et al., 1996; Guasch et al., 1998; Dorigo et al., 2004; Morin et al., 2009). Rimet, (2012) reviewed the status of diatoms as indicators of river pollution and found that species-level studies were more common than genus-level studies; indeed, community, assemblage, group and taxa level studies were more frequent than genus level-studies.

Ecological assessments at finer taxonomic levels are very useful. For example, the species *Eunotia exigua* and *Achnanthidium minutissium* are tolerant of acidic and heavy metal contaminated waters, i.e., occurring at AMD sites (DeNicola, 2000). Similarly, several diatom species are regularly reported in lotic environments exposed to toxic events, and these species are considered as indicators of organic matter (Palmer, 1969), pesticides (Debenest et al. 2010; Rimet and Bouchez, 2011; Roubeix et al., 2011, 2012) and metal pollution. Such tolerant taxa include *Achnanthidium minutissimum*, *Fragilaria* spp., *Nitzschia palea* and *Ulnaria ulna* (previously *Synedra ulna*) (Palmer, 1969; Ruggiu et al., 1998; Gold et al., 2002; Passy, 2007; Morin et al., 2008a,b; da Silva et al., 2009; Debenest et al., 2010; Rimet and Bouchez, 2011; Luís et al., 2011; Roubeix et al., 2011b; Morin et al., 2012; Roubeix et al., 2012; Pandey and Bergey, 2016).

This short literature review illustrates that diatoms show varied response to different types of contaminants. These responses may vary depending on the biogeographical region of study (Smol and Glew, 1992), in part because of taxonomic ambiguity of some widely distributed species (e.g., *Achnanthidium minutissimum*, *Nitzschia fonticola*, *Sellaphora pupula*, *Caloneis* sp., *Nitzschia* sp. and *Gomphonema* sp.) (Mann and Droop, 1996; Potapova et al., 2004; Trobajo et al., 2006; Potapova and

Hamilton, 2007; Trobajo et al., 2013; Pandey et al., 2016). Although diatom checklists and taxonomic ratings are regionally available for assessing the water quality (especially nutrient enrichment) in waterbodies (Palmer, 1969; Van Dam et al.,1994), no such well-developed resources exist for assessing heavy metal stress and organic contaminations, which is an urgent need.

2.4. Life-forms and ecological guilds

Diatoms have different strategies that resist or tolerate environmental stresses, such as grazing, flow disturbance, and nutrient resource limitation, which may differentially affect different life-forms: benthic, planktonic, mobility, colonial, tube-dwelling, stalked and adnate. Additionally, some species can be considered pioneers (early colonists), whereas others may be late successional species (Rimet and Bouchez, 2011). In contrast to these life forms, ecological guilds comprises group of species that live in same environment and exploit the same resources but are adapted in different ways to abiotic factors (Passy, 2007; Rimet and Bouchez, 2011; B-Béres et al., 2014). These biological traits provide additional information about the structure and architecture of biofilms.

Sporadic attempts have been made to explore life history characteristics and morphological growth forms of diatoms against natural (Passy and Larson, 2011; Gottschalk and Kahlert, 2012; Stenger-Kovács et al., 2013; B-Béres et al., 2014; Svensson et al., 2014) and anthropogenic (Passy, 2007; Berthon et al., 2011; Larras et al., 2014b) disturbances in fluvial ecosystems. The need to incorporate these parameters in the ecotoxicological studies has been regularly advocated (Medley and Clements, 1998; Rimet and Bouchez, 2011; Elias et al., 2015). Physico-chemical changes within fluvial

447 biofilms produce different successional patterns. According to Passy and Larson (2011)
448 algal colonization or succession is result of stresses; driven primarily by nutrient supply
449 and secondarily by current velocity of laboratory stream. On the other hand,
450 anthropogenic disturbances also have significant effects in terms of changing the
451 successional patterns of diatom species in the community, especially under heavy metal
452 contamination. For example, Medley and Clements (1998) found that small and adnate
453 (pioneer) diatom species, such as *Achnanthidium minutissimum* and *Fragilaria*
454 *vaucheriae*, dominate in the streams polluted with Zn. Field studies of periphytic diatom
455 communities collected from acid mine drainage areas regularly show the dominance of
456 pioneer diatom taxa, especially *Achnanthidium minutissimum* (Cattaneo et al., 2004;
457 Lavoie et al., 2012; Cantonati et al., 2014; Pandey et al., 2016; Luís et al., 2011; Morin et
458 al., 2012). Pandey et al. (2015) reported dominance of pioneer forms in lab cultured
459 diatom communities under Cu and Zn stress (100 ppm). In comparison to the heavy metal
460 stress, diatom biological metrices were more studied under organic contamination (such
461 as, diuron, metolachlor, acetochlor, azoxystrobin and tebuconazole). For example, Rimet
462 and Bouchez (2011) reported dominance of motile, low-profile and mucous tube forms of
463 diatoms under herbicides (diuron) and fungicides (azoxystrobin, tebuconazole) exposure
464 at environmental concentrations in lotic mesocosm experiments. Similarly, Roubeix et al.
465 (2012) reported the dominance of pioneer or low profile guilds (with *Achnanthidium*
466 *minutissimum*, *Cocconeis placentula* and *Planothidium rostratum*) and motile forms or
467 guilds (*Nitzschia palea*) in the periphytic diatom communities exposed to metolachlor
468 and acetochlor. In contrast, pesticides, including chloroacetanilides, modify the 3-D
469 structure of more complex (multispecies) biofilms by reducing biofilm thickness and the

distribution of the life forms; i.e., replacement of sensitive diatom species like *Achnanthidium minutissimum* or *Eolimna minima* (pioneer forms or low profile guild) with more tolerant species like *Nitzschia lanceolata* (motile forms) under metolachlor exposure (Roubeix et al., 2011a). Similarly, Ricart et al. (2009) showed that diuron modified the composition of periphytic diatom communities. After 1 month at a concentration of 7 ppm, *F. capucina* (high profile) was eliminated, but remained in control conditions (though at very low abundance), the abundance of *A. minutissimum* (low profile) was reduced and *Nitzschia palea* (motile guild) appeared indifferent to the presence of diuron. Recently, Larras et al. (2014b) reported that natural seasonal variation of diatom diversity (and so of sensitivity) significantly affect the community response to mixture of herbicides, as protective threshold for the herbicides mixture obtained in the winter community was not found same for the summer community based on their structural parameters. Thus, a significant difference was examined in terms of diatom community shift under heavy metal stress and organic contamination (herbicides or pesticides) namely, heavy metal stress resulted in more pioneer life forms or low profile guilds whereas organic contamination promoted motile forms.

Recognizing the roles of both life-forms and ecological guilds within biofilms enables better understanding of species-environment relationships and interactions (Passy, 2007; Passy and Larson, 2011; Berthon et al., 2011; Rimet and Bouchez, 2011; B-Béres et al., 2014). The major advantage of incorporating one or both biological metrics into ecotoxicological studies is their ease of use because there are so few categories in each metric. With few categories and application to all biofilms, these biological metrics often display robust and predictable responses to ecological gradients.

493 Biological metrics are often characteristic of the genus level for diatoms and therefore are
494 easy to use for routine monitoring purposes. These diatom metrics transform complex
495 ecological information at species level into a clearer dataset with more robust trends and
496 more easily testable hypotheses (Potapova and Charles, 2007; Rimet and Bouchez, 2011;
497 Rimet et al., 2012). Thus, biological metrics should be incorporated into the suite of
498 diatom metrics used in effective water quality bioassessment in rivers. Indeed, Medley
499 and Clements (1998) suggested incorporating periphyton community responses to
500 pollutants in terms of life-history traits, ecological strategies, and morphological forms as
501 a tool for water quality assessment.

502 **3. New endpoints**

503 *3.1. Alterations in cell integrity*

504 *3.1.1. Nuclear anomalies*

505 According to Bidle and Falkowski (2004), planktonic photosynthetic microorganisms
506 undergo programmed cell death (PCD) in response to environmental stress. Chromatin
507 condensation, migration to the edge of the nuclear membrane and fragmentation are the
508 chief morphological markers of PCD (Bidle and Falkowski, 2004). Very few reports
509 indicate how intracellular organelles, more specifically the nucleus of live diatoms, react
510 to different environmental and anthropogenic disturbances (Coombs et al., 1968; Cassotti
511 et al., 2005). To the best of our knowledge, Desai et al. (2006) is the first report of
512 genotoxic effects of metals in diatoms. Using comet assays on *Chaetoceros tenuissimus*,
513 the authors found that an elevated cadmium level damaged the nuclear material in cells
514 and that the degree of injury increased with progressive exposure. Debenest et al. (2008)
515 studied the effect of the herbicide maleic hydrazide on a freshwater benthic diatom

community in the laboratory by exposing the community to three herbicide concentrations, using an exposure time of 6h, followed by a 24h recovery period. After the maleic hydrazide treatment, a dose-dependent increase in nuclear alterations was observed (abnormal nucleus location, micronucleus, multinuclear cell or disruption of the nuclear membrane) and the difference between the control and highest dose of herbicide-treated samples was statistically significant (Fig. 1). Similarly, Licursi and Gómez (2013) reported alteration in nuclear region (abnormal nucleus location and nuclear membrane damage) in the epipsammic diatoms *Fallacia pygmaea* and *Navicula novaesiberica* after 7 days of Cr (IV) (chromium) treatment, but the difference was not statistically significant after 3 days of Cr treatment. Effects of chromium exposure differ among taxa, as no nuclear alterations were found in *Nitzschia palea* after Cr (IV) treatment (Fig. 1). Investigation of nuclear abnormalities in diatoms reveals the effects of pollutants (metals and herbicides) on the microproducers forming the basal trophic level of in fluvial ecosystems, which helps in understanding mechanisms of change in biofilm composition, especially increasing dominance of the most tolerant diatom species, as well factors that might lead to morphological anomalies in diatom frustules (Debenest et al., 2008; Licursi and Gómez, 2013).

3.1.2. Alteration in the cell membrane and cytoplasmic content

Diatoms are well known for their transparent silica frustules and, as a result, it is very easy to observe the intracellular changes (in chloroplast, cytoplasm, lipid bodies and vacuoles) under light microscopy. Alterations of cell membrane of diatoms are used to determine whether the examined cell is alive or dead (Radchenko and Il'yash, 2006; Manoylov, 2014). Morin et al. (2012) reported that metal toxicity on diatoms is linked to

539 different steps in the circulation of the toxicant across the membrane (especially uptake
540 mechanisms) and inside the cell, inducing perturbations in the normal functioning of
541 structural/functional intracellular components. Percent alteration in cell membranes was
542 calculated by measuring the changes in the perimeter of the cell membrane with respect
543 to the control specimens by using Motic (Motic, BML series, Hong Kong) or Image J
544 software (Fig. 2). Alterations was rated on the scale: 0 % denoted intact cell membrane
545 state, 25, 50, 75, and 100% denoted altered membrane state in less than one-quarter, a
546 quarter to a half, a half to three-quarters, and more than three-quarters of the live diatom
547 cells, respectively. Franklin et al. (2006) reviewed the status of programmed cell death in
548 phytoplankton ecology and advocated the use of cell viability as a health indicator.
549 Membrane integrity indicates the viability of live diatom cells and, as such, can be used
550 to indicate health in studies of species succession and food-web structure (Veldhuis et al.,
551 2001). Drum (1966) studied the degree to which plasmolysis in the anterior and posterior
552 ends of the protoplast affect cell adhesion and movement by the diatom *Pleurosigma*
553 *angulatum*. Bidle and Falkowski (2004) reviewed the status of cell death in planktonic
554 and photosynthetic microorganisms and concluded that under adverse environmental
555 conditions (nutrient deprivation, intense light, excessive salt concentrations or oxidative
556 stress), a wide variety of phytoplankton undergo apoptosis (programmed cell death), with
557 early symptoms including cell shrinkage. Chang et al. (2011) quantified cell integrity of
558 the diatom *Nitzschia palea* and the cyanobacterium *Microcystis aeruginosa* before and
559 after β-cyclocitral addition and they found that cells of *N. palea* ruptured at a much lower
560 concentration (5-10 ppm) of β-cyclocitral than in *M. aeruginosa* (200-1000 ppm).
561 Armbrecht et al. (2014) studied cell death and aggregate formation in a giant diatom

562 *Coscinodiscus wailesii* and reported autolysis in the cytoplasm of *C. wailesii* under Si and
563 N deficiency. In a laboratory experiment with *Ditylum brightwelli* and *Chaetoceros*
564 *calcitrans*, Veldhuis et al. (2001) found correlations among changes in membrane
565 permeability, photosynthetic activity, pigmentation and growth. In this study, fully
566 disintegrated cell membranes were considered non-viable. Wei et al. (2014) found that a
567 large blank space appeared between the plasma membrane and the cell wall in Cu treated
568 cells (*Phaeodactylum tricornutum*), indicating that the plasma membrane was damaged
569 and separated from the cell wall. Compared to the control, the mitochondria and nuclei in
570 the Cu-treated cells were swollen and their membranes appeared smeared. More recently,
571 Morin et al. (2016) found that in the lower Matanza-Riachuelo basin (Argentina), strong
572 symptoms of eutrophication and high amounts of organic matter and heavy metals can be
573 diagnosed through high percentages of cytological abnormalities and thus advocated the
574 need for incorporating cytological abnormalities (cytoplasmic content impaired) in
575 diatoms for the detection of changes of the water quality associated with toxic pollution.
576 This testing methodology of using cell membrane and other visible cytological
577 features to relate diatom health and, possibly, the ecological status of fluvial ecosystems
578 with the effects of toxins is relatively quick and takes little expertise in diatom taxonomy
579 (Pandey et al., 2014). Cytoplasmic alterations or autolysis of cytoplasmic content (a
580 viability assay) (comparing images of controls and treated samples; treated samples
581 showed contracted or irregular shapes compared to the control) adds a new and important
582 approach in addressing the physiological condition within a single population and thereby
583 provides information on the health status of diatom communities, which can be a tool for
584 both ecotoxicological and bioassessment studies relating to diatom biology.

585 *3.1.3. Alteration in chloroplasts (shape, size, color and number)*

586 In diatoms, chloroplasts intactness is rarely considered as a tool for ecological
587 bioassessment or for ecotoxicological assessments (Arini et al., 2012b,c). Sporadic
588 attempts have been made to establish live (with intact chloroplast) and dead (with empty
589 frustules) diatoms (Fig. 2) as a tool for bioassessment and the ecological health of
590 waterbodies (Gillet et al., 2009 and 2011). Generally, smaller chloroplast volume and less
591 thylakoid surface density were observed in diatoms under light and nutrient stress (Rosen
592 and Lowe, 1984; Janseen et al., 2001). Lommer et al. (2012) reported shrinkage of the
593 chloroplast system in iron-limited *Thalassiosira oceanica* cells. They found that Fe
594 limited cells had a reduced number of two chloroplasts, instead of the normal four. Renzi
595 et al. (2014) also reported alteration in the photosynthetic complex (by using confocal
596 and optical microscopy along with cell fluorescence) as a sensitive and powerful early
597 warning tool for evaluating sub-lethal effects of Cu, Zn and methylene blue active
598 substance (MBAS). Arini et al. (2012b,c) reported that under Cd and Zn toxicity showed
599 no significant effect on the health of diatom cells assessed in terms of presence and
600 absence of chloroplasts. However, under organic contamination, the effect on chloroplast
601 was more apparent. For example, Wood et al. (2014) developed an excellent toxicity test
602 for diatom cells by using the intactness of stained chloroplasts for assessing the toxicity
603 of atrazine over a period of 48 hours. Cells were classified depending on the condition of
604 the stained chloroplast i.e., if it appeared more than 50 % intact then it was classed as a
605 healthy cell, and if the chloroplast was < 50% intact or absent then it was classed as
606 unhealthy. In this study they found that exposure to their highest treatment of atrazine
607 (500 $\mu g\ L^{-1}$) produced significant declines in healthy cells of the most sensitive genera:

608 *Gomphonema* declined by 74%, *Amphora* by 62%, *Cymbella* by 54% and *Ulnaria* by
609 34%, compared to control levels. In contrast, the genera, *Eunotia, Achnanthidium* and
610 *Navicula*, had no statistically significant decline in cell health. By using the same
611 methodology (intactness of chloroplast),Wood et al. (2016) reported that natural benthic
612 diatom communities respond to eight common herbicides (atrazine, simazine,
613 hexazinone, tebuthiuron, diuron, MCPA, 2,4-D and glyphosate) in which the most
614 sensitive taxa were *Gomphonema* spp., *Encyonema gracilis* and *Navicula cryptotenella*
615 was the most tolerant to herbicide exposure. There was no significant effect of the
616 different herbicide modes of action at the community level. In several studies, alterations
617 in the diatom chloroplast morphology was clearly observed under different stresses, such
618 as, heavy metals (Licursi and Gómez, 2013; Pandey and Bergey, 2016) and herbicides
619 (Debenest et al., 2008).

620 Alteration in diatoms chloroplast morphology (shape, size and number) is a rapid
621 and simple toxicity testing method to obtain sensitivity data for multiple taxa within a
622 natural benthic diatom community in a relatively short period of time, warranting further
623 development of chloroplast metrics as an assessment tool. Note, however, that the
624 number and shape of chloroplasts may vary between species.

625 *3.3. Lipid bodies*

626 Different classes of algae are characterized by the type of food reserve stored inside their
627 cells (Barsanti et al., 2008). Diatoms (Bacillariophyceae) are unique in storing lipids,
628 which occur in intracellular lipid bodies (LBs), as a reserve food material (Figs. 3 and 4).
629 In diatoms, LBs become more prominent (in terms of number as well as in size) under
630 various types of environmental and anthropogenic perturbations (Ramachandra et al.

2009; Hildebrand et al. 2012) (Tables 1 and 2). Increased oil reserves may aid cell survival during unfavorable conditions. In an early observation, Evans (1960) reported that *Pinnularia biceps* f. *minutissima* cells along pond edges that had large oil globules survived desiccation better than cells with fewer lipids. Cells lacking oil globules sometimes plasmolysed and were unable to recover when put back into a liquid medium.

LB inductions are apparent under nitrogen starvation (Jiang et al., 2012; Guerra et al., 2013), which may also lead to a lower growth rate, an association meaning that reducing nitrogen may not be a good mechanism for increasing lipid yield (Hildebrand et al., 2012; Frada et al., 2013). In the same context, Julius and Theriot (2007) reported that under environmental stress, LB induction in diatom cells is associated with modification in chloroplast number. They also proposed that the chlorophyll to lipid ratio could be used as a tool to measure the health of diatom cells.

In contrast to the effects of nutrients, the effects of heavy metal stress on LBs is relatively unexplored (Pandey et al., 2015). Joux-Arab et al. (2000) reported higher lipid content in the diatom *Haslea ostrearia* under Cu stress in comparison to the control treatment in a laboratory study, but the difference was not statistically significant. Similarly, Lelong et al. (2013) described increased lipid content in Cu-starved cells of *Pseudo-nitzschia*, but no effect was observed in severe Fe-limited *Pseudo-nitzschia* cells. In contrast, mild Fe limitation produced a 100 % increase in lipid content but under Cu and Fe co-limitation, lipid content (per cell and per unit biovolume) decreased slightly. More recently, Pandey et al. (2015) reported induction of LBs (increased number and size) in a lab cultured phytoplanktonic community under Cu and Zn stress, which was also associated with deformities in the silica frustules of live diatoms. Similarly, Pandey

654 and Bergey (2016) reported higher LBs (number and biovolume) at mining sites severely
655 polluted with Cu and Zn. The number and size of LBs have also been explored as a stress
656 indicator in other algal classes, such as cyanobacteria (Peramuna et al., 2014), green
657 algae (Andrade et al., 2004; Liu et al., 2008; Wang et al., 2009) and dinoflagellates
658 (Weng et al., 2014).

659 The mechanism causing induction of lipid bodies in diatoms is not yet known. LB
660 formation is associated with the degradation or alteration of the photosynthetic apparatus
661 of diatoms (Julius and Theriot, 2007; Lommer et al., 2012; d'Ippolito et al., 2015). Lipid
662 bodies may also be a site for accumulating heavy metals (Pb and Cu) by forming lipid
663 complexes with the toxicants (Lombardi and Vieira, 1999, 2000). Lipids are used as both
664 a reserve food material and as a buoyancy organelle; buoyancy that counters the heavy
665 weight of the silica frustules and aids in the maintenance of a depth in the water column
666 appropriate for accessing light and nutrients. Buoyancy due to lipid bodies has also been
667 reported in the planktonic green alga *Botryococcus* floating on the surface of a small lake
668 (Belcher, 1968).

669 Induction of lipid bodies in diatoms apparently has multiple functions, including
670 use in combating stress. Walsby and Reynolds (1980) concluded that the reduction in
671 density due to intracellular lipid accumulation contributes to a reduced rate of sinking in
672 diatoms, but they are also of the view that buoyancy-regulation is not the primary
673 function of lipid bodies. Significant induction of lipids (40% of the cell volume) inside
674 live diatom cells can indicate its poor health i.e., increase in LB volume with decrease in
675 chloroplast volume (Liang et al., 2015; Gautam et al., 2017). Alternatively, Wang et al.
676 (2013) reported that induction of LBs in live benthic diatoms assisted their movement.

Thus, lipid body induction indicates not only an improved energy capturing capacity of diatoms, but can also act as a buoyancy regulator, as a reserve food material (Smetacek, 2001), an aid in diatom movement (Wang et al., 2013), reservoir for toxicants and as an energy storing body that can be utilized in recovering from different types of environmental and anthropogenic stresses. Lipid bodies and their induction in living diatoms form a very promising basis for easy and rapid ecological assessments as well as for biomonitoring of fluvial ecosystem.

3.4. Alteration in frustule size

Diatoms are well known for their robust, species-specific, ornamented silica frustules, which are dependably replicated from generation to generation (Falasco et al., 2009). However, these silica frustules are occasionally susceptible to alteration under various types of environmental (Winder et al., 2009; Svensson et al., 2014) and anthropogenic stress. These alterations include the production of various morphological forms (phenotypic plasticity), size reduction and even frustule deformation (Kociolek and Stoermer, 2010; Cox et al., 2012; Morin et al., 2012). According to Kociolek and Stoermer (2010), variation in diatom cell wall morphology can be either size-dependent or size-free. In the same context, Snoeijs et al. (2002) and Busse and Snoeijs (2003) found that small and large diatom species residing in the same benthic community respond differently to environmental variation (Si, nutrients, phosphate, salinity and wave action). Size reduction is a normal feature of diatoms due to their vegetative mode of reproduction (Laney et al., 2012), in which one cell of the original size and a slightly smaller cell are produced during each cell division. Variation in size may be adaptive or circumstantial (Kociolek and Stoermer, 2010). An example of an environmental effect

700 was reported by Trobajo et al. (2011), who found that saline concentration significantly
701 affected diatom width (and stria density) in *Nitzschia frustulum* and *N. pusilla*, but had no
702 effect on valve length in any of the five studied species.

703 Heavy metal enrichment is a major factor intensifying the naturally occurring
704 phenomenon of size reduction in diatom frustules (Fig. 5; Table 3) and can result in
705 significant reduction in diatom size (Morin et al. 2012; Cantonati et al., 2014). Cattaneo
706 et al. (2004) reported significant size reduction in four diatom species (*Achnanthidim*
707 *minutissimum*, *Asterionella formosa*, *Brachysira vitrea* and *Tabellaria flocculosa*)
708 collected from the heavy metal (Cd, Cu, Fe, Pb and Zn) polluted sediments of a
709 watershed of Lac Dufault (Québec, Canada) and Morin and Coste (2006) reported
710 significant reduction in two abundant diatom species, *Gomphonema parvulum* and
711 *Nitzschia palea*, collected from metal (Cd and Zn) polluted sites (Joanis and Usine) of
712 Riou Mort and Riou Viou streams of South West France. Similarly, Luís et al. (2011)
713 reported > 60 % size reduction in *Brachysira vitrea* at the abandoned mining area in
714 Portugal, which is highly contaminated with heavy metals (in both sediments and surface
715 water). Barral-Fraga et al. (2016) reported significant size reduction in different diatom
716 species (*Amphipleura pellucida*, *Nitzschia dissipata*, *Nitzschia fonticola* and *Nitzschia*
717 *palea*) treated with arsenic in a laboratory experiment.

718 The exact mechanism of intensified size reduction in diatom frustules is uncertain,
719 but according to Morin et al. (2012), size reduction can be the result of a higher cell
720 division rate that is inherent to organisms inhabiting stressed ecosystems. Climate
721 warming in conjunction with increased organic loads could also facilitate increased
722 phosphate availability (Wilhelm and Adrian, 2008), that could result in a decrease in

723 dissolved Si:P and decrease in light availability, potential favouring smaller diatoms,
724 especially in late spring and summer (Kilham et al., 1996; Finkel et al., 2009). The
725 phenomenon of significant reduction in diatom frustule size has potential use in
726 ecotoxicological studies, but further work is needed. Size reduction occurs more
727 frequently than cell deformity (see next section), but it is difficult to segregate the effects
728 of stress-related reduction from the natural tendency of diatom populations to get smaller
729 and smaller with successive cell divisions. Deformity and size reduction in diatoms often
730 occur together and if we able to establish that significant size reduction is due to stress, as
731 has been done previously (Cattaneo et al., 2004), then size reduction might prove to be a
732 valuable biomonitoring tool.

733 Larger cell size in diatoms has been reported under different types of stress, such
734 as light (Olson et al., 1986), temperature (Montagnes et al., 2001), long-term culturing
735 (Rose and Cox, 2013) and heavy metals (Stauber and Florence, 1987; Levy et al., 2008),
736 although some studies do not indicate a clear size trend (Montagnes, 2001; Levy et al.
737 2007). Greater cell size has been reported mainly in marine centric diatoms (*Aulacoseira*
738 sp. *Biddulphia aurita*, *Cosinodiscus* sp., *Lauderia borealis*, *P. tricornutum*, *Skeletonema*
739 *costatum*, *Stephanopyxis turris*, *Thalassiosira weissflogii*, and *T. psuedonana*). In
740 converse, pennate diatoms in any aquatic ecosystem are rarely reported as having larger
741 than expected cell size (Reavie and Barbiero, 2013).

742 Diatom size and changes in size (and surface area) might be associated to nutrient
743 absorption, especially in nutrient-limited (oligotrophic) environments. Increasing
744 frustules length or diameter is also considered an adaptation to reduce sinking rates
745 (Miklasz and Denny, 2010), which may be enhanced by a longer ice-free season and

746 stronger late-summer stratification. According to Kerrigan et al. (2015), climate warming
747 and increases in the duration of the growing season and stratified period may allow
748 greater accumulation of nutrients during transient mixing events in the spring and fall
749 overturns (Kilham et al., 1996), which may perhaps favor larger-sized diatom species in
750 the community. The relationship between cell size and genome size is of significant
751 importance in diatoms. For example, Koester et al. (2010) examined two isolated
752 population of *Ditylum brightwellii* from New Zealand and found that their increased sizes
753 are directly related with amount of DNA content in the cells. Similarly, van Tol et al.
754 (2016) recently reported a symbiotic association between bacteria (*Croceibacter*
755 *atlanticus*) and the marine diatom *Thalassiosira pseudonana*, which resulted in an
756 inhibition of cell division and induces the mean size of diatom cells to become longer.
757 These changes could be explained by an absence of cytokinesis that causes individual *T.*
758 *pseudonana* cells to elongate, accumulate more plastids and become polyploid.
759 Frustule morphology may differ strikingly between the largest and smallest cells
760 in a size series of a single species, or even a single clone (Cox et al., 2012), which is a
761 challenge to community analysis based on diatom size. Furthermore, size ambiguity in
762 diatoms illustrates that molecular bioassessment simply based on a DNA code may be
763 inadequate to describe diatoms because the expression of proteins depends on the unique
764 set of environmental conditions in which algae live and affects diatom shapes,
765 adaptations, and survival (Will and Rubinoff, 2004; Sluys, 2013). In addition, the identity
766 of most diatom taxa in the reference libraries of genetic sequences has not been
767 rigorously evaluated, so the reference library taxonomy may not be accurate for
768 molecular bioassessment (Manoylov, 2014).

769 *3.5. Morphological deformities*

770 Morphological abnormalities in diatom frustules are globally assessed under various
771 environmental (aging, crowding, tides, mechanical pressure, low current velocity,
772 drought, light intensity, temperature, moisture, salinity, pH, long-term culture, Si and
773 nutrients) and anthropogenic (heavy metals, herbicides and pesticides) disturbances
774 (Falasco et al., 2009) (Figs. 6 and 7). The proportion of valve abnormalities was inferred
775 directly from the taxonomical counts, by determining the percentage of individuals that
776 had unusual shape and/or ornamentation of the frustule. Deformations in diatoms were
777 first correlated with physical variables such as temperature, current velocity, flow and
778 rainfall, rather than with chemical variables (Gómez and Licursi, 2003). Recent studies
779 reported significant relationships between anthropogenic stresses (herbicides; Roubeix et
780 al., 2011b and heavy metals; Pandey et al., 2014) and abnormalities in diatom frustules.
781 For example, under natural conditions, the percent of deformed frustules was
782 significantly lower (0.35 %) than under heavy metal stress (1.0-4.0 %) (Falasco et al.,
783 2009; Morin et al., 2012; Pandey et al., 2014), whereas organic contamination was
784 associated with an intermediate deformity percent of between 0.32 and 1.5 % (Morin et
785 al., 2009; Debenest et al., 2008; Roubeix et al., 2011a).

786 Deformities in diatoms are often not associated with species that dominant under
787 various anthropogenic stresses. For example, Roubeix et al. (2011a) reported a higher
788 percent of deformity in a sub-dominant diatom species, i.e., *Surirella angusta* under
789 diuron exposure. Similarly, Duong et al. (2008) reported deformities in the diatom
790 species *Fragilaria capucina*, *Gomphonema parvulum* and *Ulnaria ulna*, which was
791 numerically average or sub-dominant in abundance. Dziengo-Czaja et al. (2008) also

792 reported deformities in diatom species that were not prevalent in the nutrient (phosphorus
793 and nitrite) enriched (organic matters) habitat. In contrast, deformities in dominant
794 diatom species (*Achnanthes minutissima* and *Brachysira vitrea*) occurred in waterbodies
795 contaminated with heavy metals (AMD sites) (Cattaneo et al., 1998, 2004; Luís et al.,
796 2011).

797 Araphid and monoraphid forms are more susceptible to deformation than other
798 morphological forms. For example, in the diatom genera *Achnanthidium* (*Achnanthidium*
799 *minutissimum*), *Fragilaria* (*Fragilaria capucina* and *F. rumpens*) and *Ulnaria* (*Ulnaria*
800 *ulna*), deformed frustules have been associated with various types of anthropogenic
801 perturbations (Gold et al., 2002, 2003b; Nunes et al., 2003; Duong et al., 2008; Morin et
802 al., 2008a; Roubeix et al., 2011b; Lavoie et al., 2012; Cantonati et al., 2014; Pandey et
803 al., 2014, 2015; Pandey and Bergey, 2016). In contrast, Morin et al. (2008b) reported
804 more deformities in raphid forms (66 %) than araphid ones (33%) in a freshwater biofilm
805 exposed to Cd. However, under herbicide contamination, deformities were equally shared
806 mainly between raphid and araphid forms (Debenest et al., 2008; Roubeix et al., 2011b).

807 The relationship of diatom size to deformity has been debated. Morphological
808 abnormalities are often missed in small diatoms when diatoms are viewed with light
809 microscopy (Morin et al., 2008c; Manoylov, 2014). Studies using scanning electron
810 microscopy indicate that small diatoms are as susceptible to deformities as larger species
811 (Morin et al., 2008c). However, according to Lavoie et al. (2012), the lower percentage of
812 deformed frustules in the small diatom *Achnanthidium minutissimum* may signify that
813 small species are less susceptible to morphological deformations, although Cantonati et
814 al. (2014) extensively studied (and found) deformities in this species from 8 different

sites in Europe and Canada. Furthermore, the length/width ratio of diatom species also plays a role, especially in araphid forms, as species with higher length/width ratios are generally more prone to deformation under stress than other shapes of diatom frustules.

Another issue to consider is the low percentage of deformed frustules even under stress conditions, which is the major concern in assessing the use of deformities as a biomonitoring tool. Incorrect identification of diatom species and the lack of proper training to discriminate deformed frustules from normal ones are main reasons for lower counts of deformity in diatom assemblages. In addition, permanent slide preparation results in an inability to examine all sides of individual diatoms for deformities. Examining all four views (two valve and two girdle views) of diatom frustules would almost certainly increase the percent of deformed cells counted in samples with deformities.

In diatoms, deformities are quantified as percentages by counting 500 or 1000 frustules in the community. In order to make deformities more informative, Falasco et al. (2009) broadly classified deformities into 8 types, a classification refined by other researchers (Arini et al., 2012b,c; Pandey et al., 2014, 2015), culminating into four types i.e., deformities in valve (type I), striae (type II), raphe (type III) and mixed types (type IV) (Table S1). All four types of deformity were examined under heavy metal contamination by Cu and Zn in the field and laboratory conditions (Pandey et al., 2014, 2015) (Table 4). Reports of deformities in diatoms exposed to heavy metal pollution indicate primarily of Type 1 (valve outline) and Type 2 (striae) deformities. Gómez and Licursi (2003) reported the occurrence of all four types of deformities (valve, striations, raphe and mixed) in the diatom species *Pinnularia gibba* in a periphytic biofilm in an

838 area with Cu and Zn contamination. Arini et al. (2012a,b) similarly reported prevalence
839 of deformed valves (type 1), deformed striations (type 2) and mixed deformities (type 4)
840 in the periphytic diatom community contaminated with Cd and Zn, and that type 3
841 (deformed raphe) deformities were present in low percentages. In field studies, Pandey et
842 al. (2014) and Pandey and Bergey (2016) found that deformed raphes (type 3) were more
843 prevalent under Cu stress than other deformities, but that deformed striations (type 2) and
844 mixed deformities (type 4) were more prevalent under Zn stress. Laboratory experiments
845 using planktonic communities produced similar results [i.e., Cu stress lead to higher
846 prevalence of deformed raphes (type 3), whereas Zn exposure resulted in a higher
847 prevalence of deformed striae (type 2)]. Arini et al. (2013) ran an experiment that
848 described the development and persistence of deformities in a population of the diatom
849 *Planothidium frequentissimum* within a Cd-impacted population, in terms of the viability
850 of deformed frustules, their reproduction capabilities, and the time for the population to
851 return to normal forms after the cessation of pollution. During the 21 days of Cd
852 treatment, deformities of the striae and mixed anomalies appeared first, followed by
853 alterations in central region and valve outlines. After an additional 28 days with no Cd
854 exposure, a reduction in deformed frustules was observed but deformities were still
855 present. Deformations of the striae appeared to be the most sustainable phenotype, since
856 they were still significantly higher than in reference cultures at the end of the
857 decontamination phase for the previously exposed the Cd cultures. Thus, deformities
858 remain after numerous cell divisions.

859 Another issue to consider is the low percentage of deformed frustules even under
860 stress conditions, which is the major concern in the use of deformities as a biomonitoring

tool. Another issue is that reports of higher incidences of deformed frustules are often from field sites that are concomitantly contaminated with different toxicants. Thus, it is not always possible to attribute deformities to any particular contaminant or group of contaminants (Falasco et al., 2009), which ultimately leads to weak statistical relationships between deformities and the contaminants (Cattaneo et al., 2004). In situ studies using diffusing substrates, such as nutrient diffusing substrates (NDSs) (Pringle, 1990) or metal diffusing substrates (MDSs) (Pandey et al., 2014), are an excellent tool to assess the effect of single toxicants or combinations of toxicants on frustule abnormalities in benthic diatoms.

4. Conclusion and future prospective

Traditional community structural metrics using diatoms (for bioassessment and ecotoxicological studies) can provide effective diagnostic information about fluvial ecosystems. However, the extensive time (and financial) requirements, necessity of expertise in diatom taxonomy and the need for statistical validation means that the use of structural metrics are often not feasible at the local level, and can sometimes make the use of diatom metrics unpopular. Conversely, recently developed non-taxonomical metrics effectively deal with these shortcomings, as these metrics are relatively quick, easy, cost-effective, reproducible and are based on globally accepted protocols. Furthermore, non-taxonomic metrics allow for comparisons between different sites and even geographical locations (countries, continents etc.), as they are independent of the taxonomic similarity or differences. Thus, refinement of current protocols, especially through incorporation of new metrics, is needed to improve bioassessment and

ecotoxicological assessment studies of diatoms and for developing efficient and effective biomonitoring strategies.

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Conflict of interest
The authors declare that they have no conflict of interest.

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Legend for the figures

Fig. S1. Photographic documentation (SEM, scanning electron microscopy) of 12 sensitive and 13 tolerant diatom species reported from contaminated waterbodies of Korea. Superscripts "S" showed the sensitive diatom species while the others are tolerant. For SEM investigation, diatom samples were fixed in 2.5% glutaldehyde for 5 h and then washed with a 0.1 M phosphate buffer solution. The samples were then dehydrated through a series of 30%, 50%, 70%, 80%, 90%, 95% and 100% acetone. Cleaned material was mounted on a stub on which Hexamethyledisilazane was added, sputter-coated with Gold + Palladium and examined using a JEOL/JSM-7001F, Japan. (The results presented were obtained by the authors of the present ms).

Fig. 1. Alterations in cell integrity as nuclear anomalies. The effect of maleic hydrazide (MH) on the diatom genera (*Nitzschia* (a), *Gomphonema* (b) and *Navicula* (c)) with normal (N), abnormal nuclear location (ANL), fragmented nucleus (FN), micronucleus (MN) and broken nucleus membrane (BNM) (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). Scale bar- 10 µm. Source- Debenest et al., (2008). The effect of hexavalent chromium on the cells of *Fallacia pymaea* (d) and *Navicula novaesiberica* (e) with normal (N), abnormal nuclear location (ANL), fragmented nucleus (FN), micronucleus (MN) and broken nucleus membrane (BNM) (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). Scale bar- 10µm. Source-Licursi and Gómez, (2013).

Fig. 2. Alteration in the cell membrane and cytoplamic content. (a) Percent decrease in the photosynthetic apparatus (PDPA) of live *Navicula*, (b) Percent decrease in the photosynthetic apparatus (PDPA) of live *Amphora* under Cu stress under laboratory conditions. Scale bar- 8µm. (The results presented were obtained by the authors of the present ms).

Fig. 3. Lipid bodies visualization in the periphytic community dominated with diatoms and stained with BODIPY fluorescent dye. Green fluorescence is BODIPY staining of neutral lipids, red is chlorophyll fluorescence. Scale bar- 10 µm. (The results presented were obtained by the authors of the present ms).

Fig. 4. Light micrographs of lipid bodies in 29 diatom species. (1) *Navicula veneta*, (2) *N. gregaria*, (3) N. *decussis*, (4) *N. schoenfeldii* (5) *N. capitatoradiata*, (6) *N. cincta*, (7) *N. accomoda*, (7) *N. gregaria*, (8) *N. phyllepta*, (9) *Craticula cuspidata*, (10) *Gyrosigma exilis*, (11) *G. nodiferum*, (12) *Navicula incertata*, (13) *Achnanthidium exiguum*, (14) *Pinnularia subcapitata*, (15) *Tryblionella debilis*, (16) *Cocconeis placentula*, (17) *Nitzschia palea*, (18) *N. linearis*, (19) *N. amphibia*, (20) *N. sigmoidea*, (21) *N. sigmoidea*, (21) *Bacillaria paradoxa*, (22) *Nitzschia intermedia*, (23) *N. frustulum*, (24) *N. filiformis*, (25) *N. inconspicua*, (26) *Tryblionella apiculata*, (27) *Plagiotropis lepidotropis*, (28) *Haslea ostrearia*, (28) *Bacillaria paradoxa* and (29) *Gyrosigma spencerii*. Scale bar-5µm. (The results presented were obtained by the authors of the present ms).

Fig. 5. Photographic documentation of alteration in frustule size in 14 commonly occurring diatom species under different environmental and anthropogenic disturbances.(1) *Navicula salinarium,* (2) *Navicula recens,* (3) *Diploneis interrupta,* (4) *Surirella stalagama,* (5) *Tryblionella coarctata,* (6) *Diploneis elliptica,* (7) *Surirella robusta,* (8) *Surirella gemma,* (9) *Meloneis mimallis* var. *mimallis,* (10) *Encyonema* sp., (11) *Amphora* sp., (12) *Cocconeis placentula,* (13) *Meloneis akytos* and (14) *M. mimallis* var. *zephyria*. Scale bar- 5µm. (The results presented were obtained by the authors of the present ms).

Fig. 6. Morphological deformities observed in living diatom frustules examined under different types of environmental and anthropogenic perturbations. (1) *Navicula veneta,* (2) *Nitzschia linearis,* (3) *Nitzschia amphibia,* (4) *Fragilaria capucina,* (5) *Diatoma vulgare,* (6) *Diatoma vulgare,* (7) *Nitzschia filiformis,* (8) *Gomphonema pseudoaugur,* (9) *Fragilaria capucina* and (10) *Ulnaria ulna*. First frustules in each species is normal while rest ones are deformed. Scale bar- 8µm. Source- Pandey et al., 2015; Pandey and Bergey, 2016; Gautam et al., 2017.

Fig. 7. Normal (first frustules) and Deformed frustules in 29 diatom species examined under different types of environmental and anthropogenic purturbations. (1) *Achnanthidium breviceps,* (2) *Mastogloia smithii,* (3) *Luticola muticopsis,* (4) *Nitzschia compressa,* (5) *Planothidium frequentissimum,* (6) *Diatoma vulgare,* (7) *Diatoma vulgare,* (8) *Diatoma* sp., (9) *Tryblionella apiculata,* (10) *Cymbella tumida,* (11) *Cocconeis placentula,* (12) *Eolimna subminuscula,* (13) *Achnanthidium exiguum,* (14) *Fragilaria* sp., (15) *Tabularia fasciculata,* (16) *Fragilaria capucina,* (17) *Nitzschia clausii,* (18) *Ulnaria ulna,* (19) *Fragilariforma bicapitata,* (20) *Brachysira microcephala,* (21) *Caloneis bacillum,* (22) *Caloneis bacillum* (Girdle view), (23) *Nitzschia palea,* (24) *Ulnaria ulna,* (25) *Cymbella turgida,* (26) *Gomphonema pseudoaugur,* (27) *Ulnaria ulna,* (28) *Brachysira brebissonii* and (29) *Gomphonema parvulum.* First frustules in each species is normal while rest ones are defromed. Scale bar- 7µm. (The results presented were obtained by the authors of the present ms).

Table 1. Biovolume and number of lipid bodies in five diatom species exposed to metals (Cu and Zn; 100 µg l^{-1}) under laboratory conditions. See Fig. 7 (14, 1, 6, 20 and 19). Source: Pandey et al., 2015.

Diatom species	***Biovolume (µm^3 Cell^{-1}) of lipid bodies (number of lipid bodies)**			**% contribution of lipid bodies to total cell volume under metal stress**		
	Control	**Cu**	**Zn**	**Control**	**Cu**	**Zn**
Achnanthidium exiguum	2* (1)	6* (2)-23(2)	4* (1)-16(2)	7	20-77	13-53
Navicula gregaria	4 (2)	20(4)-90(4)	13(3)-68(4)	2	9-41	6-31
Navicula recens	3 (2)	12(2)-67(2)	12(2)-61(2)	1.5	6-34	6-31
Nitzschia amphibia	4 (2)	52(6)-165(6)	45(4)-145(4)	1.2	16-50	14-44
Nitzschia linearis	4 (0)	16(6)-126(5)	16(5)-126(5)	1.3	5-40	5-40

Table 2. Number of lipid bodies and % biovolume contribution of lipid bodies per cell in three common diatom species examined at the metalliferous sites (Khetri and Zawar) of Rajasthan, India. See Fig. 7 (15, 19, 21 and 22). Source: Pandey and Bergey, 2016.

Diatom species	**No. of lipid bodies (diameter in µm)**		**% contribution of lipid bodies to total cell volume under metal stress**	
	Control	**Metal stress (Cu, Zn)**	**Control**	**Metal stress (Cu, Zn)**
Pinnularia subcapitata	2 (0.1-0.5)	4 (1-3)	2-5	15-60
Nitzschia linearis	4 (0.1-0.32)	4 (1-2.8)	4-8	14-43
Nitzschia sigmoidea	5 (0.1-0.2)	10 (1-2)	2-5	12-25

Table 3- Investigation of % change in cell length in 14 different diatom species under environmental and anthropogenic disturbances. The diatom samples were collected from various contaminated (nutrient and heavy metals) waterbodies of Korea during 2014-2016. Percent change in length is percent loss of length relative to the longest cell measured.

	Diatom species	**No. of frustules examined**	**% change in length**
1.	*Cocconeis placentula*	50	0-25
2.	*Diploneis elliptica*	50	0-85
3.	*Diploneis interrupta*	50	5-58
4.	*Encyonema minutum*	50	0-55
5.	*Meloneis akytos*	50	5-80
6.	*Meloneis gorgis*	50	0-90
7.	*Meloneis mimallis* var. *mimallis*	50	10-80
8.	*Meloneis mimallis* var. *zephyria*	50	0-10
9.	*Navicula recens*	50	15-55
10.	*Navicula salinarium*	50	10-60
11.	*Surirella gemma*	50	5-65
12.	*Surirella robusta*	50	0-40
13.	*Surirella stalagma*	50	5-65
14.	*Tryblionella coarctata*	50	0-60

Table 4- Relative proportions (%) of various deformities examined under in situ (45-165 µg cm^{-2} d^{-1} for Cu; 42-150 µg cm^{-2} d^{-1} for Zn in 14 days) and laboratory (100 µg l^{-1} after 7 days) condition under Cu and Zn stress (separately) in the periphytic diatoms (Pandey et al., 2014, 2015; Pandey and Bergey, 2016).

	In situ		**Lab**	
	Control	*Stressed*	*Control*	*Stressed*
Total deformity (%)	0.1	3.0	2	10
Type 1 (Valve)	70	76	80	40
Type 2 (Striation)	30	5	20	20
Type 3 (Raphe)	0	17	0	20
Type 4 (Mixed)	0	2	0	20

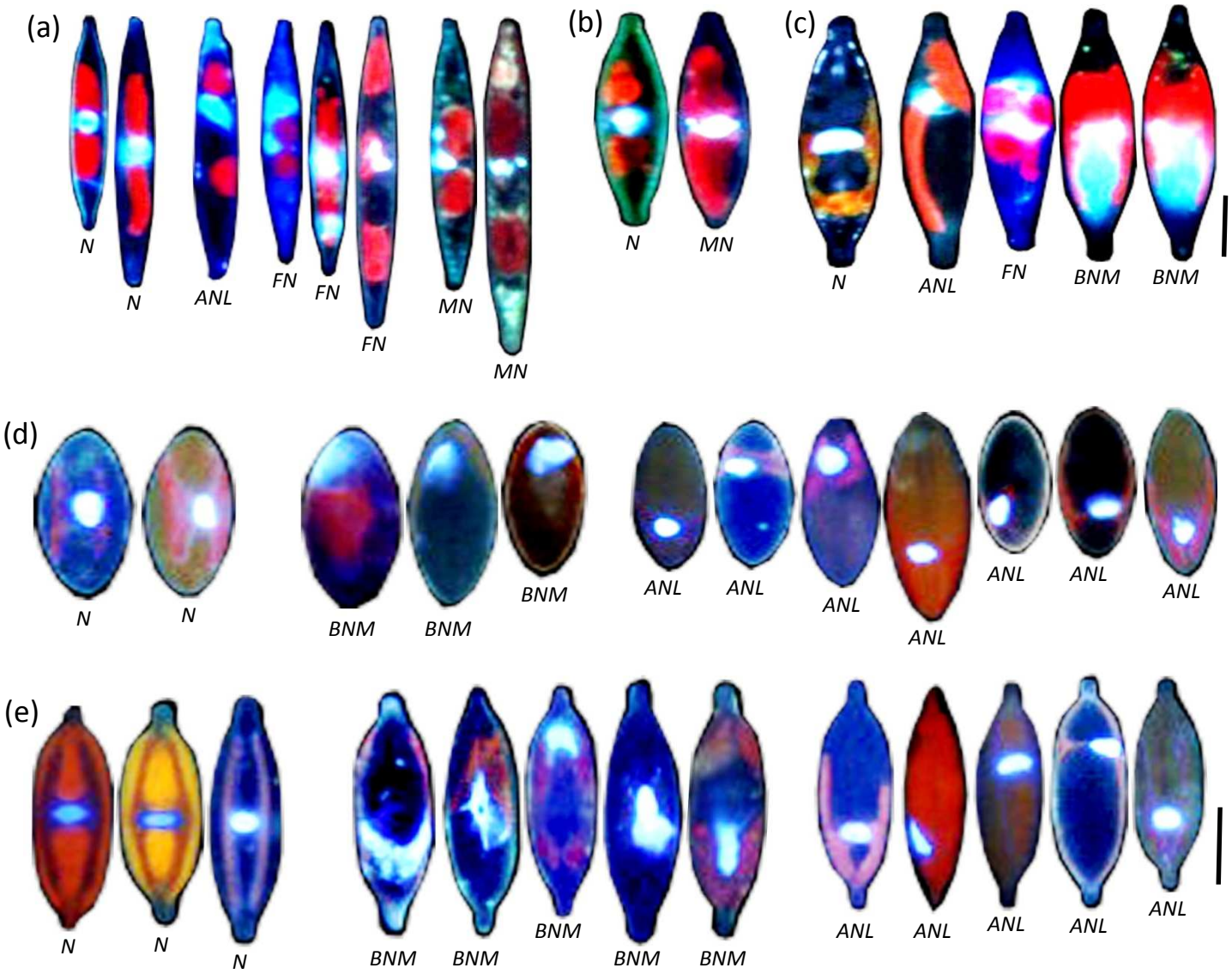

Figure 1. Alterations in cell integrity as nuclear anomalies. The effect of maleic hydrazide (MH) on the diatom genera (*Nitzschia* (a), *Gomphonema* (b) and *Navicula* (c)) with normal (N), abnormal nuclear location (ANL), fragmented nucleus (FN), micronucleus (MN) and broken nucleus membrane (BNM) (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). Scale bar- 10 µm. Source- Debenest et al., (2008). The effect of hexavalent chromium on the cells of *Fallacia pymaea* (d) and *Navicula novaesiberica* (e) with normal (N), abnormal nuclear location (ANL), fragmented nucleus (FN), micronucleus (MN) and broken nucleus membrane (BNM) (nucleus stained in blue with Hoescht 33342, chloroplasts appear in red). Scale bar- 10µm. Source-Licursi and Gómez, (2013).

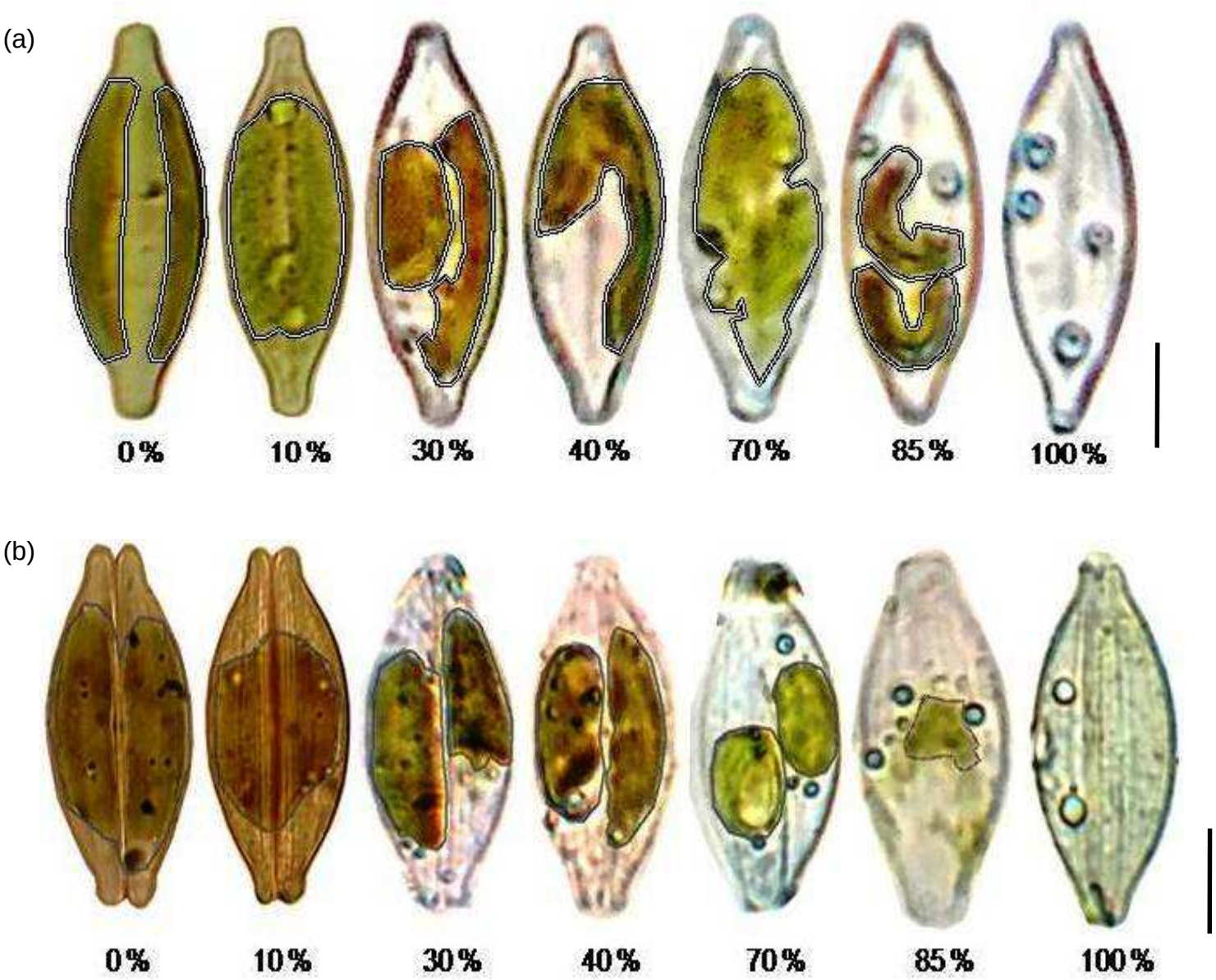

Fig. 2. Alteration in the cell membrane and cytoplasmic content. (a) Percent decrease in the photosynthetic apparatus (PDPA) of live *Navicula*, (b) Percent decrease in the photosynthetic apparatus (PDPA) of live *Amphora* under Cu stress under laboratory conditions. Scale bar- 8µm. (The results presented were obtained by the authors of the present ms).

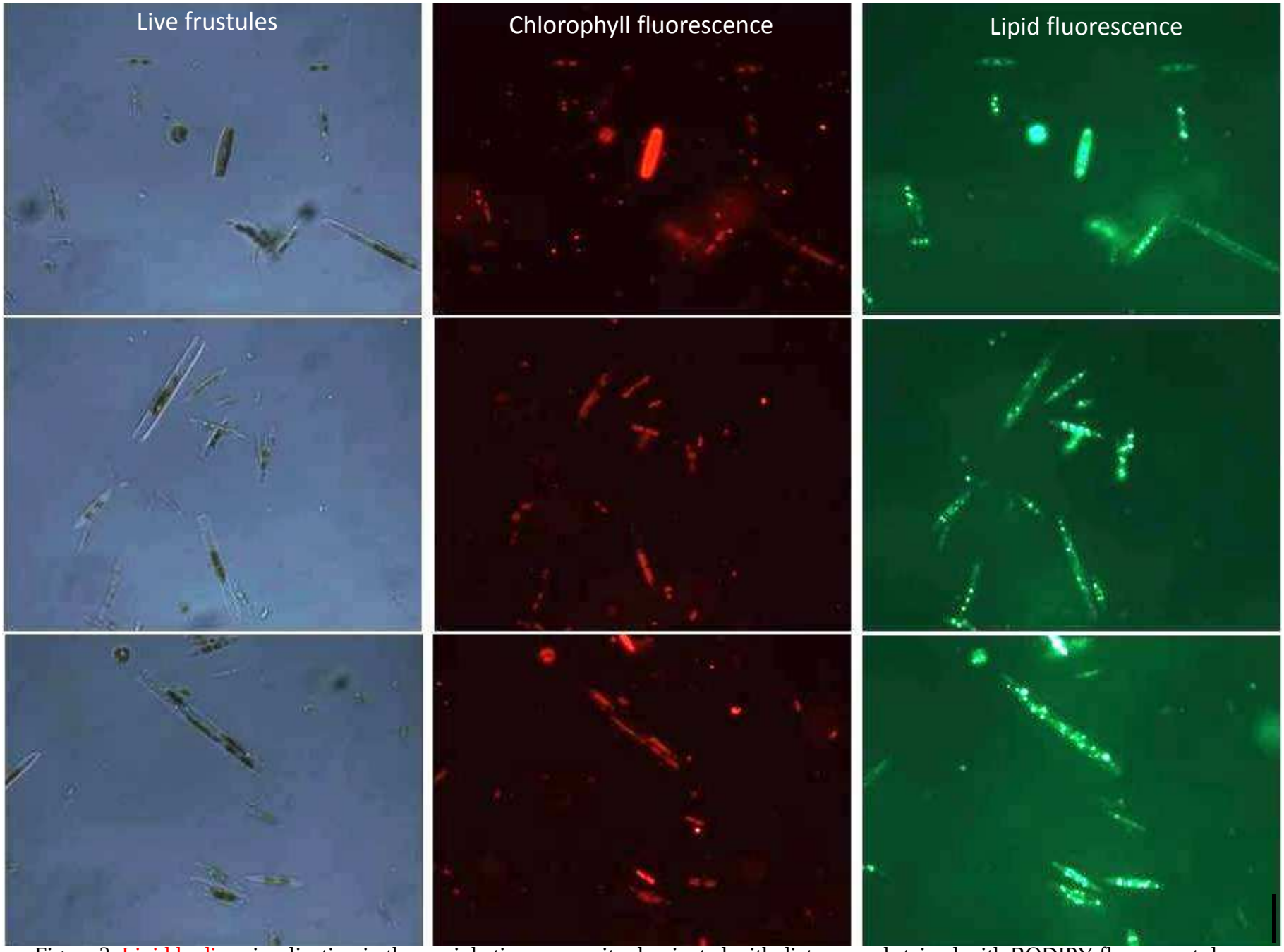

Figure 3. Lipid bodies visualization in the periphytic community dominated with diatoms and stained with BODIPY fluorescent dye. Green fluorescence is BODIPY staining of neutral lipids, red is chlorophyll autofluorescence . Scale bar- 10 μm. (The results presented were obtained by the authors of the present ms).

Figure 4. Light micrographs of lipid bodies in 29 diatom species. (1) *Navicula veneta*, (2) *N. gregaria*, (3) N. *decussis*, (4) *N. schoenfeldii* (5) *N. capitatoradiata*, (6) *N. cincta*, (7) *N. accomoda*, (7) *N. gregaria*, (8) *N. phyllepta*, (9) *Craticula cuspidata*, (10) *Gyrosigma exilis*, (11) *G. nodiferum*, (12) *Navicula incertata*, (13) *Achnanthidium exiguum*, (14) *Pinnularia subcapitata*, (15) *Tryblionella debilis*, (16) *Cocconeis placentula*, (17) *Nitzschia palea*, (18) *N. linearis*, (19) *N. amphibia*, (20) *N. sigmoidea*, (21) *N. sigmoidea*, (21) *Bacillaria paradoxa*, (22) *Nitzschia intermedia*, (23) *N. frustulum*, (24) *N. filiformis*, (25) *N. inconspicua*, (26) *Tryblionella apiculata*, (27) *Plagiotropis lepidotropis*, (28) *Haslea ostrearia*, (28) *Bacillaria paradoxa* and (29) *Gyrosigma spencerii*. Scale bar-5µm. (The results presented were obtained by the authors of the present ms).

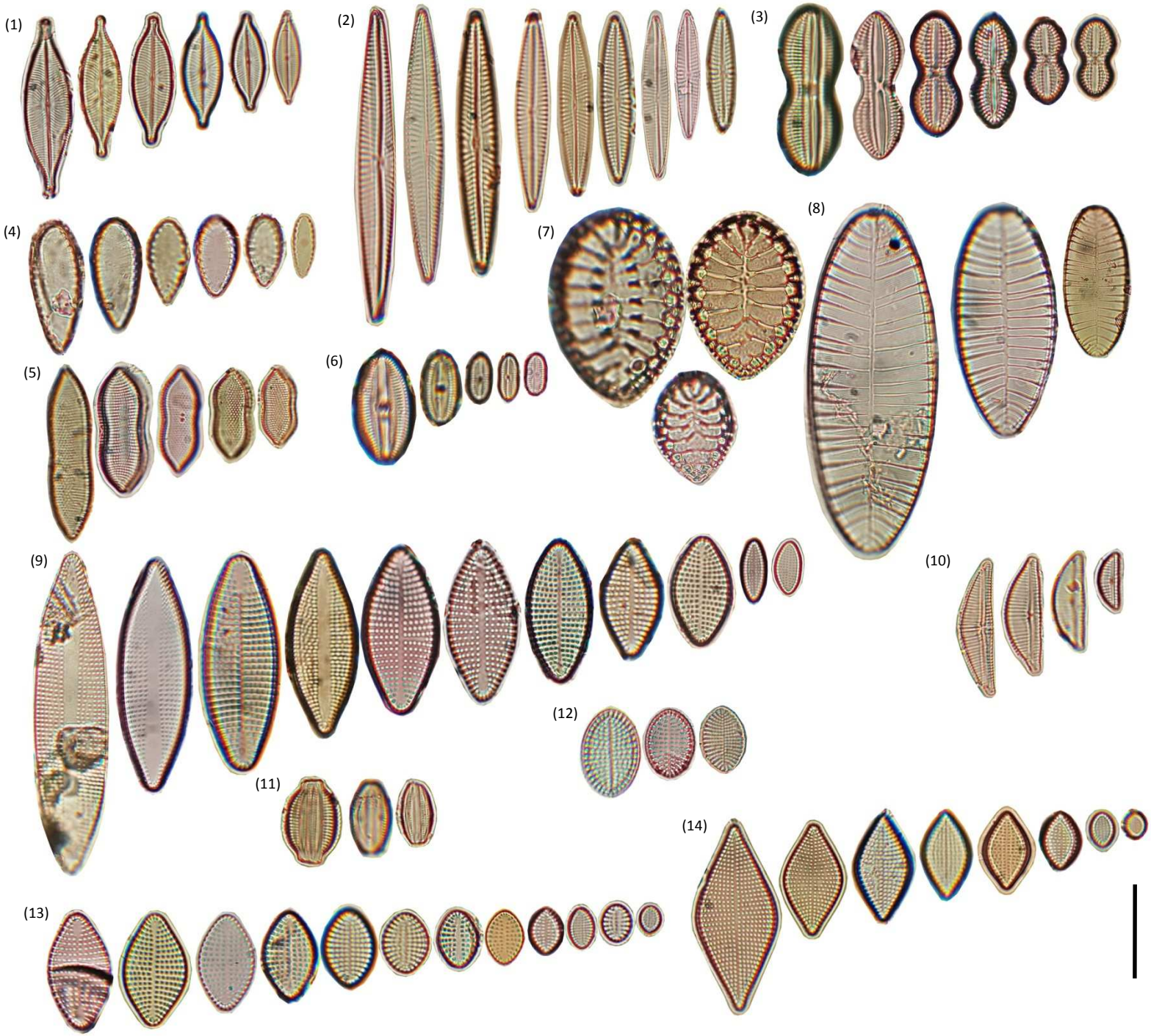

Figure 5. Photographic documentation of alteration in frustule size in 14 commonly occurring diatom species under different environmental and anthropogenic disturbances.(1) *Navicula salinarium*, (2) *Navicula recens*, (3) *Diploneis interrupta*, (4) *Surirella stalagama*, (5) *Tryblionella coarctata*, (6) *Diploneis elliptica*, (7) *Surirella robusta*, (8) *Surirella gemma*, (9) *Meloneis mimallis* var. *mimallis*, (10) *Encyonema* sp., (11) *Amphora* sp., (12) *Cocconeis placentula*, (13) *Meloneis akytos* and (14) *M. mimallis* var. *zephyria*. Scale bar- 5µm. (The results presented were obtained by the authors of the present ms).

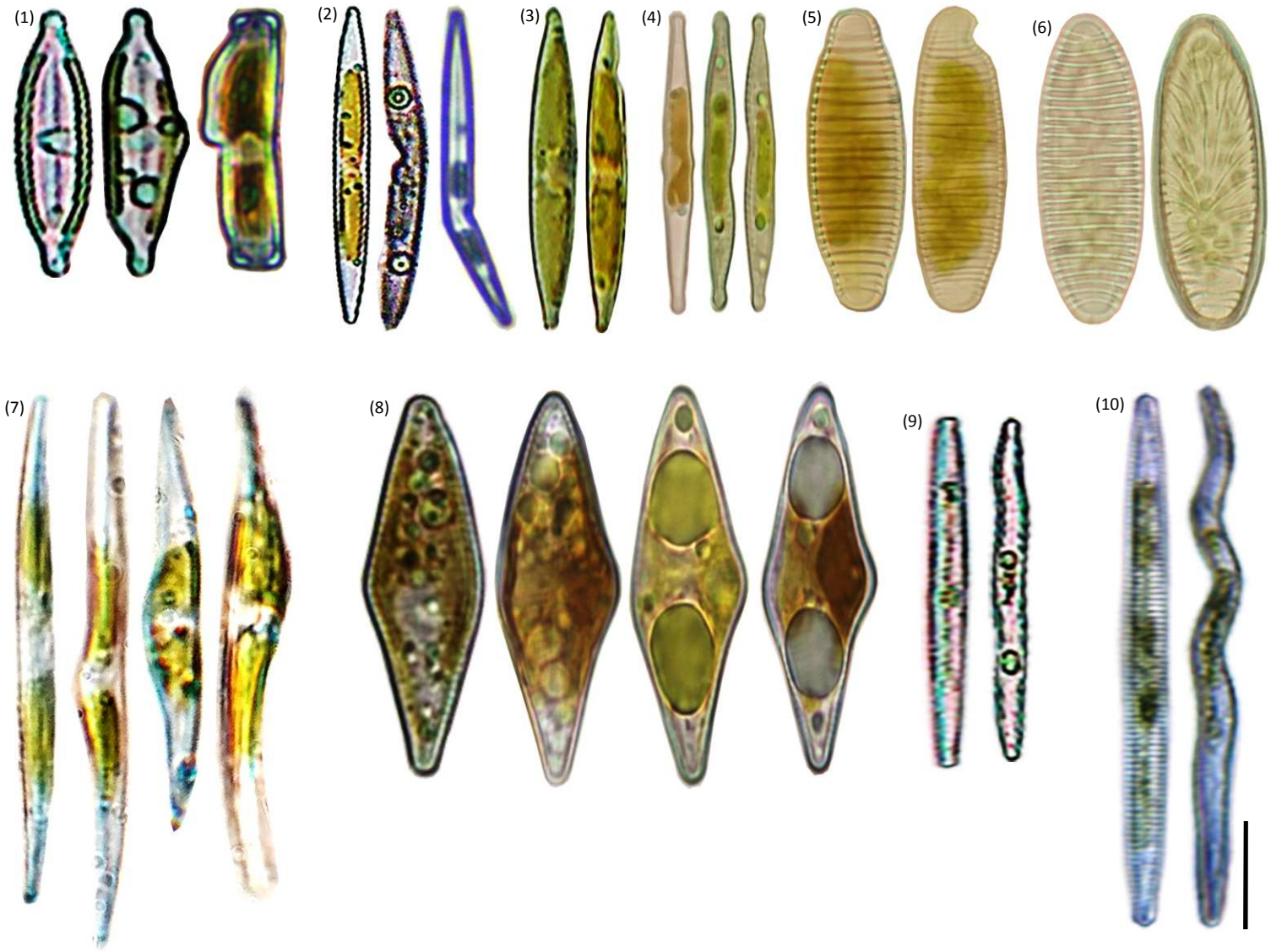

Figure 6. Morphological deformities observed in living diatom frustules examined under different types of environmental and anthropogenic perturbations. (1) *Navicula veneta*, (2) *Nitzschia linearis*, (3) *Nitzschia amphibia*, (4) *Fragilaria capucina*, (5) *Diatoma vulgare*, (6) *Diatoma vulgare*, (7) *Nitzschia filiformis*, (8) *Gomphonema pseudoaugur*, (9) *Fragilaria capucina* and (10) *Ulnaria ulna*. First frustules in each species is normal while rest ones are deformed. Scale bar- 8µm. Source- Pandey et al., 2015; Pandey and Bergey, 2016; Gautam et al., 2017.

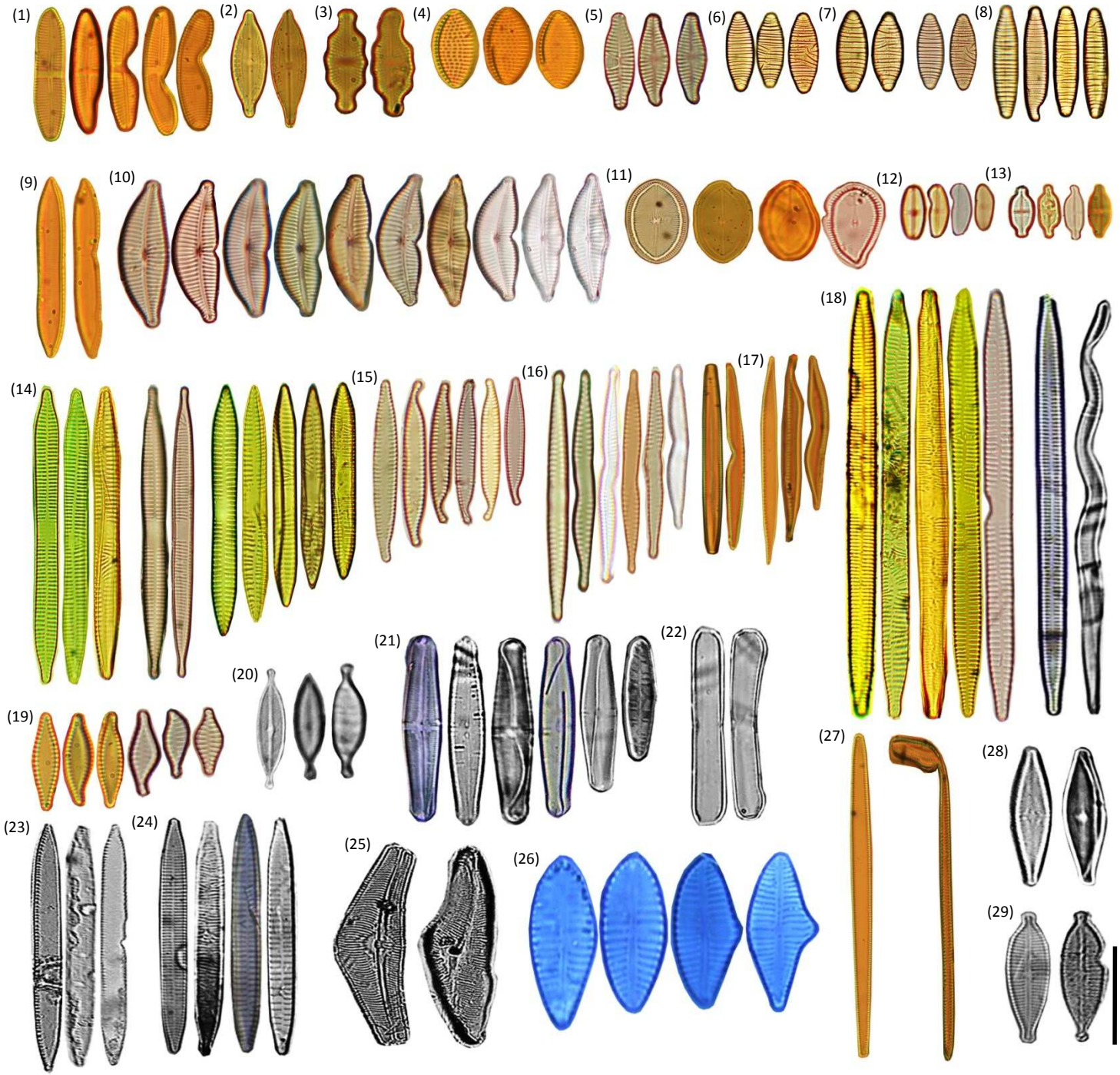

Figure 7. Normal (first frustules) and Deformed frustules in 29 diatom species examined under different types of environmental and anthropogenic purturbations. (1) *Achnanthidium breviceps*, (2) *Mastogloia smithii*, (3) *Luticola muticopsis*, (4) *Nitzschia compressa*, (5) *Planothidium frequentissimum*, (6) *Diatoma vulgare*, (7) *Diatoma vulgare*, (8) *Diatoma* sp., (9) *Tryblionella apiculata*, (10) *Cymbella tumida*, (11) *Cocconeis placentula*, (12) *Eolimna subminuscula*, (13) *Achnanthidium exiguum*, (14) *Fragilaria* sp., (15) *Tabularia fasciculata*, (16) *Fragilaria capucina*, (17) *Nitzschia clausii*, (18) *Ulnaria ulna*, (19) *Fragilariforma bicapitata*, (20) *Brachysira microcephala*, (21) *Caloneis bacillum*, (22) *Caloneis bacillum* (Girdle view), (23) *Nitzschia palea*, (24) *Ulnaria ulna*, (25) *Cymbella turgida*, (26) *Gomphonema pseudoaugur*, (27) *Ulnaria ulna*, (28) *Brachysira brebissonii* and (29) *Gomphonema parvulum.* First frustules in each species is normal while rest ones are defromed. Scale bar- 7µm. (The results presented were obtained by the authors of the present ms).

Highlights

1. Diatom-based taxonomical parameters were investigated under various stresses.
2. Status of new diatom endpoints was examined under various stresses.
3. New diatom endpoints are easy, quick, cheap and need less human expertise.
4. Traditional and new endpoints required for effective biomonitoring practices.