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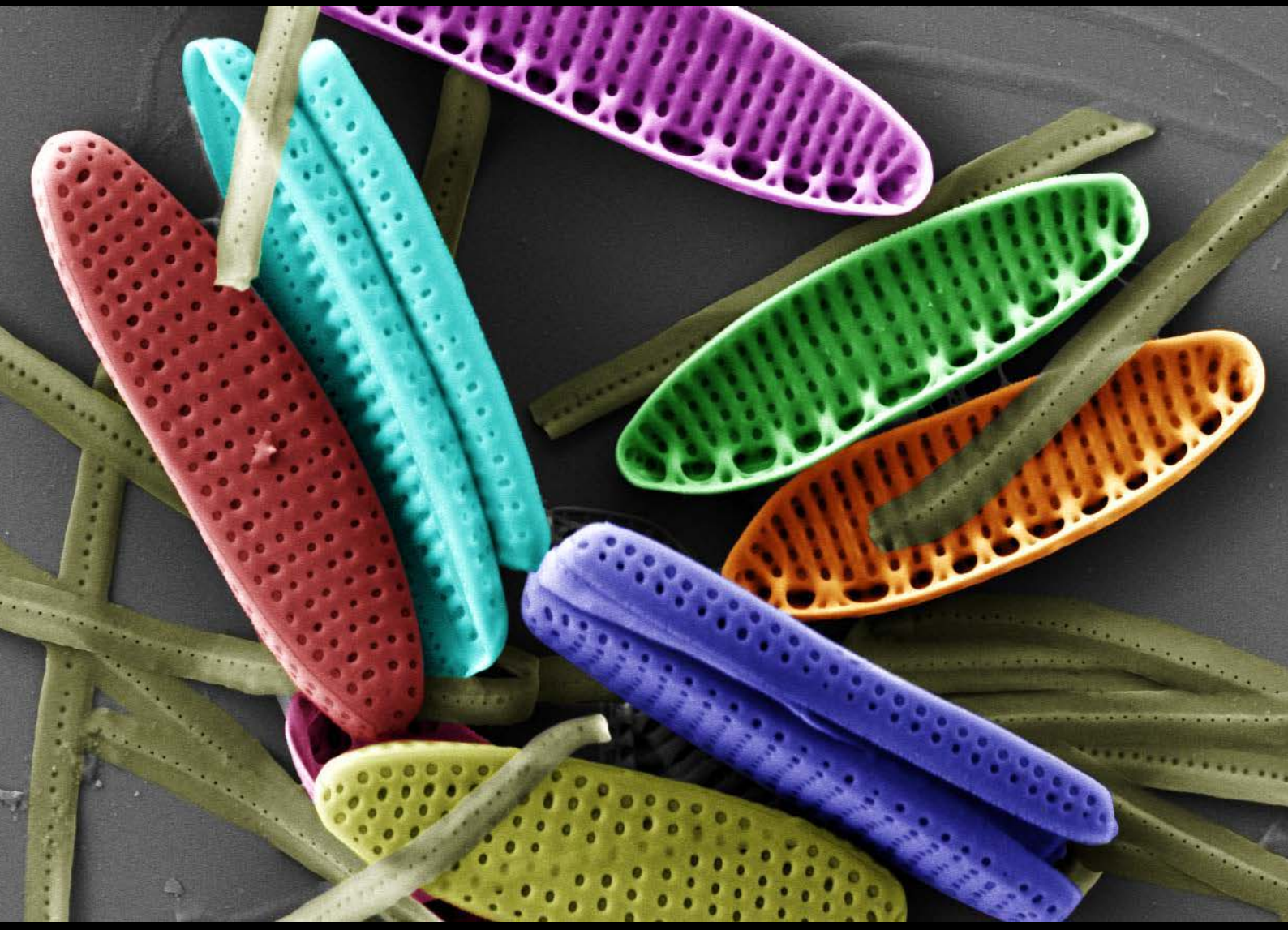

Laia Rovira Torres

2013

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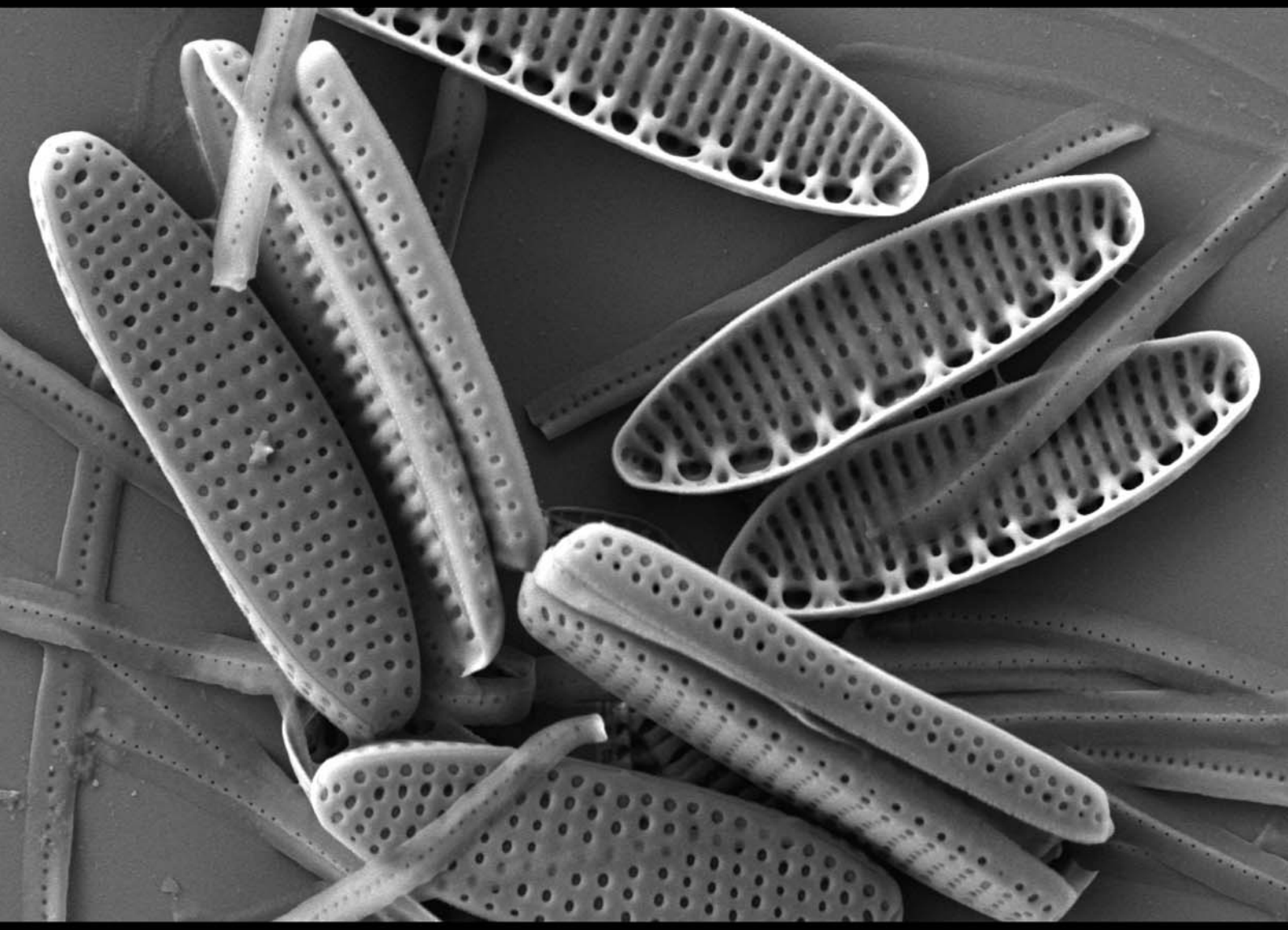
2. **Rovira, L.**, Trobajo, R. & Ibáñez, C. (2012) The use of diatom assemblages as ecological indicators in highly stratified estuaries and evaluation of existing diatom indices. **Marine Pollution Bulletin** 64, 500-511.
3. Trobajo, R., **Rovira, L.**, Mann, D.G. & Cox, E.J. (2011) Effects of salinity on growth and on valve morphology of five estuarine diatoms. **Phycological Research** 59, 83-90.
4. Trobajo, R., **Rovira, L.**, Ector, L., Morales, E., Wetzel, C. E., Kelly, M. & Mann, D.G. (2012) Morphology and identity of some ecologically important small *Nitzschia* species. **Diatom Research** 28 (1), 37-59.
5. **Rovira, L.**, Trobajo, R., Sato, S., Ibáñez, C. & Mann, D.G. Genetic and ecophysiological diversity in the diatom *Nitzschia inconspicua*. **Journal of Phycology** (under review).

Appendix:

Rovira, L., Trobajo, R. & Ibáñez, C. (2009) Periphytic diatom community in a Mediterranean salt-wedge estuary: the Ebro Estuary (NE Iberian Peninsula). **Acta Botanica Croatica** 68 (2), 285-300.

Rovira, L., Witkowski, A.,Trobajo, R., Ibáñez, C. & Ruppel, M. (2011) *Planothidium iberense* spec. nov., a new brackish diatom of the Ebro Estuary (NE Spain). **Diatom Research** 26 (1), 99-107**.**

Mann, D.G., Sato, S., **Rovira, L.**, & Trobajo, R. (2013) Paedogamy and auxosporulation in *Nitzschia* sect. *Lanceolatae* (Bacillariophyta). **Phycologia** 52 (2), 204-220.



laia.rovira.torres@gmail.com

TESI DOCTORAL

Departament d'Ecologia

UNIVERSITAT DE BARCELONA

Programa de doctorat: Ecologia Fonamental i Aplicada

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Memòria presentada per

Laia Rovira Torres

Per optar al grau de

Doctora per la Universitat de Barcelona

Barcelona, Juliol de 2013

Vist-i-plau dels directors de la tesi

Dra. Rosa Trobajo Pujadas
Investigadora del Programa
d'Ecosistemes Aquàtics
IRTA

Dr. Carles Ibáñez i Martí
Director del Programa
d'Ecosistemes Aquàtics
IRTA

Vist-i-plau de la tutora de la tesi

Dra. Isabel Muñoz Gracia
Departament d'Ecologia
Universitat de Barcelona

Als meus pares i germana

A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

Acknowledgments

Aquest és el final d'un llarg camí en el qual m'hauria perdut sense el recolzament que m'han donat tantes persones durant aquests anys.

Arribar fins aquí no hauria estat possible sense l'ajut i el suport dels meus directors durant el procés que ha comportat l'elaboració d'aquest treball. Gràcies Rosa per introduir-me al fascinant mont d'aquests microorganismes tant bells com són les diatomees, per animar-me a viatjar i a enriquir-me amb les estades i congressos que he pogut realitzar, i per tenir sempre temps per a mi. Gràcies Carles per donar-me l'oportunitat de demostrar que era capaç d'arribar fins aquí, per totes les facilitats de les quals he pogut gaudir aquests anys, i per encomanar-me la passió pel Delta i pel nostre riu Ebre. També vull agrair a la Isabel Muñoz, per ser la meva tutora i estar sempre pendent de tota la burocràcia i les novetats en les normatives. I als meus professors, sobretot a la Montse Blanch i al Xavi Parra, per l'educació rebuda i per haver-me inspirat a seguir pel camí de la biologia.

En aquesta tesis doctoral han participat diatomòlegs i científics d'arreu d'Europa, l'ajuda dels quals ha estat essencial a l'hora d'avançar en la recerca feta. Thank you David Mann for your advice and support throughout my entire thesis, for making me feel at home when in Edinburgh and allowing me to learn from you. Shinya, thanks for all the molecular discussions and the lunches with the girls, it has been very enriching working with you, I hope to visit you soon! Also to all the staff from the Royal Botanic Garden of Edinburgh for their help during my stay. Thanks to all the people that collaborated in the publications that are included in this thesis, especially to Martyn Kelly for his insatiable research on *N. inconspicua*, to Manfred Ruppel for his amazing SEM pictures and to Luc Ector for the huge amount of bibliography provided. Dziękuję, to all the people from the Palaeoceanology Unit in Szczcecin University, especially to Andrzej Witkowski for giving me the opportunity to learn and discuss the taxonomy of those amazing (and sometimes extremely complicated!) organisms, and for your encouragement in describing new species. To Maxim for all the walks, ice creams and moments shared, and to Elena for being like my second mum, for your kindness and the nice Christmas and Easter Holidays postcards that I am still receiving. To Diana for showing me the Polish nightlife and for taking the time to listen when I needed it. Des de Galicia, gracias Manel por tu paciencia con mis dudas estadísticas, por las largas y productivas conversaciones tanto en persona como por Skype y por haberme brindado la oportunidad de colaborar contigo.

Durant els congressos als que he assistit he compartit molts moments amb altres diatomòlegs que m'han ensenyat que, a part d'aprendre i enriquir-te professionalment, també hi ha temps per a passar una bona estona. Gràcies a en Lourenço, en Pieter, la Irene i la Cristina per celebrar les reunions "diatomològiques" tal com cal!

Han estat molts anys treballant a l'IRTA de Sant Carles de la Ràpita, i vull agrair a tota la gent que ha compartit el dia a dia amb mi. A la gent d'Administració, per tota la paperassa i les vegades que van haver d'enviar/rebre paquetets amb cultius, a la gent de suport, a la Pili i a la Cinta per les xerrades i els "xistes" de capvespre, a la Mari Pau, David, Isolda, Albert, Bea, María, Iñaki, Sandra, Míriam i a tota la gent amb la que he compartit tants esmorzars i estones al Sol. Gracias Anaïs por ser la mejor compañera de piso, por estar siempre dispuesta a escucharme y por tu maravillosa tartiflette! De manera especial, vull agrair a la gent d'Ecosistemes Aqüàtics, per ajudar-me tant en els mostrejos com en el laboratori o amb la bibliografia, sobretot a la Rosa Valmaña, David Mateu, Lluís, i Carme, i als meus companys de despatx, sense les bogeries i els deliris dels quals les tardes/vespres s'haurien fet interminables: Sílvia, Gloria, Fon, Núria i Rosa Andreu, espero que el destí ens torni a creuar algun dia!

El camí que porta al final d'una tesis no és sempre fàcil, i he tingut la sort de comptar amb amics i familiars que valen el seu pes en or i que m'han fet costat tant en els moments alegres com en els no tant alegres. L'amistat autèntica no entén de distància, i jo us sento tant prop com si us tingués al meu costat! Algunes d'aquestes persones ara no formarien part de la meva vida si no fos per aquesta tesis, i per això vull agrair a l'Andrea, Carmen, Josu i Marta totes les tardes a la platja, les paelles, els viatges, els concerts, els cafès, les cerveses, els xipirons, les tallarines, els Sant Joans, les excursions, i com no les nits rapitenques amb banys al mar a altes hores de la matinada i meduses incloses! Estoy segura que allá donde estemos siempre encontraremos la manera de vernos otra vez.

Altres amics porten al meu costat anys i al seu costat he crescut com a persona i sento que aquesta tesis és en part seva. A tota la gent de Llorenç, són tants anys compartits juntes! Cris, sembla que fos ahir quan vaig arribar a Llorenç i anàvem a classe juntes, i ara ja ets mare (i per tant jo tieta). Judit, quan em parlaves de la tesis mai hagués pensat que un dia jo estaria al mateix lloc que tu...i si, hi ha llum al final del túnel! A les meves nenes del Delta...Alenda, sempre recordaré les nostres cartes del principi i les sortides rapitenques del final, "quién nos ha visto y quién nos ve"! Cinta, els nostres viatges a Conil, els tintos de verano, que bé que m'han anat aquestes vacancetes. Gemma, tu sempre tant reivindicativa i amb tanta iniciativa, espero que alguna cosa se m'hagi encomanat. Són tants moments viscuts amb vosaltres que em fan falta pàgines, i n'hi hauria per escriure 5 tesis com aquesta...juntes hem (més o menys) madurat, i la nostra amistat continua intacta com el primer dia.

A la gent de Barcelona, amb la qual vaig compartir 5 anys d'estudis, de biblioteca, d'estones al bar, d'excursions, de disfresses, de "matxades", i d'anades de l'olla vàries... Gràcies Ariadna, Carlos, Elena, Francesc, Lídia i Quim per haver compartit amb mi una de les millors èpoques de la meva vida. Més recentment, a la gent de la Ràpita, en especial a Aitor i Lluch, per enriquir-me musicalment al vostre costat, esto no se para! Gracias Patri por tus clases de enología, por nuestras noches de fin de año, de jersei de renos, guitarra, cartas y Skype en diferentes continentes. Des de Edimburgo, a Bea, Lorena, Laura, Pollo, Pedrín, Estefanía,

Dacil, Paola, Jesús y a la gente de LUSH por aguantar y compartir mis locuras y hacer del frío algo pasajero, prometo no decir nunca más eso de: no puedo, quiero acabar la tesis!

Vull agrair de tot cor a la meva família pels valors inculcats i per estar al meu costat de manera incondicional. Al meu padrí, tieta Mercè, Aida, Gerard, Maria Teresa, Desi, a la meva fillola Emma, al meu iaio, prometo estar més disponible a l'Skype a partir d'ara, i ja podeu anar comprant un bitllet d'avió per venir a veure'm! A la meva mare i a la meva germana Anna...no hi ha paraules per a agrair tot el que heu fet per mi, i sense vosaltres res no tindria sentit. Anna, molta sort en la nova aventura que emprens, estic molt orgullosa de tu. Rick, thanks for being in my life these last three years, for being my strength and supporting me in every moment, I cannot imagine finishing my PhD without you by my side. To Anne and Chris, thanks for being my second family in the UK.

Per a acabar, vull agrair de manera molt especial a les persones que, malhauradament, ja no estan al meu costat, però les quals estan i estaran sempre en la meva memòria, i aquesta tesis està especialment dedicada a elles. Gràcies iaia i tieta Rosalia, pels Nadals i Pasqües en família, pel vostre amor i fer dels estius al Masroig una època inoblidable. L'últim agraïment és per a la persona més important per a mi, la que més m'ha influenciat i a la que més trobo a faltar. Papa, gràcies per sempre creure en mi, per fer-me forta i per ser el millor pare del món. M'hagués agradat que haguessis pogut compartir aquest moment amb mi, però sé que allí on estiguis estaràs orgullós de que hagi arribat fins aquí.

Contents

Directors' report

Informe dels directors de la tesi doctoral: "The ecology and taxonomy of estuarine benthic diatoms and their use as bioindicators in a highly stratified estuary (Ebro Estuary, NE Iberian Peninsula): a multidisciplinary approach" en relació a la contribució de la doctoranda i les publicacions derivades.

Els directors de la Tesi Doctoral de Laia Rovira Torres,

Informen que durant el període 2008-2013 la Laia Rovira Torres ha realitzat totes les tasques derivades de la realització de la seva tesi. Els treballs de recerca portats a terme per Laia Rovira Torres durant la seva formació com a doctoranda han donat lloc a 7 articles publicats en revistes indexades, 4 dels quals constitueixen el cos de la tesis juntament amb un manuscrit que està en procés de publicació. Els 3 restants s'han inclòs a l'Apèndix.

Certifiquem que la Laia Rovira Torres ha participat de forma molt activa en el desenvolupament dels treballs de recerca associats a cadascun dels articles, així com en la seva elaboració. En concret, la seva participació en les tasques de la tesi, sota supervisió dels directors, ha estat la següent:

- Planificació inicial dels objectius de cadascun dels treballs.
- Elaboració del disseny experimental, presa de mostres al camp, anàlisi de mostres al laboratori i realització d'experiments de laboratori.
- Creació de les bases de dades i anàlisis estadístic de les dades.
- Redacció dels articles, amb la col·laboració dels corresponents coautors.
- Redacció del document de la Tesi Doctoral.

A continuació es detalla el llistat d'articles inclosos a la tesi així com els factors d'impacte (segons el SCI de la ISI web of Knowledge) de les revistes corresponents i les tasques realitzades en els treballs en coautoria:

Rovira, L., Trobajo, R., Leira, M. & Ibáñez, C. (2012) The effects of hydrological dynamics on benthic diatom community structure in a highly stratified estuary: The case of the Ebro Estuary (Catalonia, Spain). **Estuarine, Coastal and Shelf Science** 101, 1-14. Factor d'impacte: 2.247 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de les categories 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 20 de 97 revistes, i 'Oceanography' (mediana del factor d'impacte: 1.472) sent la número 16 de 59 revistes.

Rovira, L., Trobajo, R. & Ibáñez, C. (2012) The use of diatom assemblages as ecological indicators in highly stratified estuaries and evaluation of existing diatom indices. **Marine Pollution Bulletin** 64, 500-511. Factor d'impacte: 2.503 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de les categories 'Environmental Sciences' (mediana del factor d'impacte: 1.562) sent la número 54 de 205 revistes, i 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 14 de 97 revistes.

Trobajo, R., **Rovira, L.**, Mann, D.G. & Cox, E.J. (2011) Effects of salinity on growth and on valve morphology of five estuarine diatoms. **Phycological Research** 59, 83-90. Factor d'impacte: 1.453 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de la categoria 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 44 de 97 revistes. Tasques realitzades per la doctoranda: Planificació inicial

dels objectius, presa de mostres de camp, aïllament i manteniment dels cultius monoclonals al laboratori i revisió del manuscrit.

Trobajo, R., **Rovira, L.**, Ector, L., Morales, E., Wetzel, C. E., Kelly, M. & Mann, D.G. (2012) Morphology and identity of some ecologically important small *Nitzschia* species. **Diatom Research** 28 (1), 37-59. Factor d'impacte: 0.656 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de la categoria 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 76 de 97 revistes. Tasques realitzades per la doctoranda: Planificació inicial dels objectius, col·laboració en la documentació gràfica i l'anàlisis de les espècies sota microscopia òptica i electrònica, elaboració d'una figura inclosa com a material suplementari, creació de bases de dades per a l'anàlisi estadístic i revisió del manuscrit.

Rovira, L., Trobajo, R., Sato, S., Ibáñez, C. & Mann, D.G. Genetic and ecophysiological diversity in the diatom *Nitzschia inconspicua*. **Journal of Phycology** (submitted). Factor d'impacte: 2.071 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de les categories 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 28 de 97 revistes, i 'Plant Sciences' (mediana del factor d'impacte: 1.372) sent la número 59 de 190 revistes.

Articles inclosos a l'Apèndix:

Rovira, L., Trobajo, R. & Ibáñez, C. (2009) Periphytic diatom community in a Mediterranean saltwedge estuary: the Ebro Estuary (NE Iberian Peninsula). **Acta Botanica Croatica** 68 (2), 285-300. Factor d'impacte: 0.702 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de la categoria 'Plant Sciences' (mediana del factor d'impacte: 1.372) sent la número 135 de 190 revistes.

Rovira, L., Witkowski, A.,Trobajo, R., Ibáñez, C. & Ruppel, M. (2011) *Planothidium iberense* spec. nov., a new brackish diatom of the Ebro Estuary (NE Spain). **Diatom Research** 26 (1), 99-107**.** Factor d'impacte: 0.656 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de la categoria 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 76 de 97 revistes.

Mann, D.G., Sato, S., **Rovira, L.**, & Trobajo, R. (2013) Paedogamy and auxosporulation in *Nitzschia* sect. *Lanceolatae* (Bacillariophyta). **Phycologia** 52 (2), 204-220. Factor d'impacte: 2.000 (ISI Journal Citation Report 2011). Aquesta revista es troba dins de les categories 'Marine and Freshwater Biology' (mediana del factor d'impacte: 1.474) sent la número 32 de 97 revistes, i 'Plant Sciences' (mediana del factor d'impacte: 1.372) sent la número 60 de 190 revistes. Tasques realitzades per la doctoranda: Planificació inicial dels objectius, presa de mostres de camp, aïllament i manteniment dels cultius monoclonals al laboratori, col·laboració en la redacció dels Materials i Mètodes i revisió del manuscrit.

Finalment, certifiquem que cap coautor dels articles esmentats ha utilitzat, implícitament o explícitament, la informació presentada per a l'elaboració d'una altra tesi doctoral. Informem que hem fet la revisió de l'esborrany final de la tesi doctoral per tal de ser entregada al Departament d'Ecologia de la Universitat de Barcelona.

Atentament,

Dr. Carles Ibáñez Martí
Director de Tesi

Dra. Rosa Trobajo Pujadas
Directora de Tesi

Sant Carles de la Ràpita, 28 de març de 2013

Resum de la tesi

Introducció

Estuaris: definició, classificació i avaluació del seu estat ecològic

Etimològicament, la paraula "estuari" prové del llatí "*aestuarium*", que significa "terra inundada per la marea". Des dels anys 50, s'han proposat diverses definicions i classificacions d'acord amb les característiques biològiques, geològiques, geomorfològiques, hidrològiques i químiques dels estuaris (per a una revisió ampliada consultar Ibáñez 1993; Perillo 1995). En aquest treball s'ha considerat la definició d'Ibáñez (1993), ja que és prou general com per incloure tots els tipus d'estuaris però suficientment específica per a diferenciar els estuaris d'altres masses d'aigua costaneres:

"Un estuari és un sistema fluvio-marí (engolfament o part final d'un riu) exposat a la influència mareal, i es caracteritza per una barreja variable i no necessàriament permanent (en l'escala espacial i temporal) d'aigua marina i d'aigua dolça provinent del drenatge continental."

La dinàmica hidrològica dels estuaris és una de les característiques principals que s'utilitza en les classificacions. Segons Hansen i Rattray (1966), els estuaris es poden dividir en quatre tipus:

Tipus 1: El flux net d'aigua és en direcció al mar per a tota la columna d'aigua, i el flux longitudinal de sal aigües amunt es produeix per difusió. Aquest tipus inclou la majoria dels estuaris no estratificats, però també alguns estuaris parcialment estratificats.

Tipus 2: El flux net d'aigua a la superfície és en direcció al mar i s'inverteix en profunditat. El flux longitudinal de sal es dóna tant per advecció com per difusió. Tot i que aquesta categoria inclou alguns estuaris no estratificats, la majoria dels estuaris considerats són parcialment estratificats.

Tipus 3: El flux net d'aigua és el mateix que en el tipus 2, però el flux longitudinal de sal es dóna principalment per advecció. Els fiords s'inclouen en aquest tipus, amb una estratificació permanent i moderada a causa de la profunditat de la capa d'aigua salada.

Tipus 4: L'estratificació vertical és encara més accentuada i la circulació per advecció és molt reduïda. Els estuaris pertanyents a aquest tipus són estuaris altament estratificats amb falca salina, i es localitzen normalment en mars caracteritzats per una amplitud mareal molt feble (micromarees). Alguns exemples de mars "micromareals" els trobem al Mediterrani i el Golf de Mèxic. La posició de falca salina depèn principalment del cabal dels rius, desapareixent quan el

cabal del riu és molt elevat. Alguns exemples d'estuaris amb falca salina són el Mississipí, el Roine i el nostre lloc d'estudi, l'estuari de l'Ebre (Ibáñez et al. 1997).

La presència de la falca salina divideix la columna d'aigua en una capa superior d'aigua dolça que prové del riu i una capa profunda salada provinent del mar, separades per una estreta interfase. La forta estratificació no només es pot detectar amb el gradient de salinitat, sinó també per una composició fisicoquímica i un funcionament ecològic diferent en cada capa. Mentre que la producció primària es concentra a la capa superior (és a dir, la que rep la major part de la llum i els nutrients provinents de les d'aigües fluvials), a la falca salina l'aigua pot tenir un temps de residència molt elevat i la matèria orgànica acumulada es va consumint, la qual cosa pot causar fortes condicions d'hipòxia que afavoreixen l'augment de: i) compostos d'amoni reduïts com a resultat de la desnitrificació per part de bacteris anaerobis, i ii) ortofosfats a causa de processos de solubilització (Ibáñez et al. 1995). Les condicions d'hipòxia solen ser més acusades prop del límit de la falca salina, on l'aigua pot romandre estancada durant mesos.

Tots els estuaris comparteixen un gran dinamisme a causa de l'amplitud de les seves marees o les fluctuacions de la falca salina, definint-los com a ecosistemes naturalment estressats (Elliott i Mclusky 2002; Mclusky i Elliott 2007). En el cas dels estuaris amb falca salina, la dinàmica imprevisible de la falca modificarà les característiques fisicoquímiques de la columna d'aigua (p. ex., des d'aigua oxigenada i dolça a aigües marines i anòxiques) (Ibáñez et al. 1995). El complex funcionament hidrològic dels estuaris els fa sistemes difícils d'estudiar i de modelar conceptualment (Trobajo i Sullivan 2010), però a la vegada proporcionen el cas idoni per entendre la resposta integrada dels organismes a la combinació i interacció de diferents tipus d'estrès d'origen natural i antropogènic.

En general, els estuaris es caracteritzen per ser sistemes altament productius que proporcionen una valuosa font de béns i serveis com ara les zones de cria per a peixos i espècies d'invertebrats, llocs d'alimentació i d'hivernació per a les aus, el reciclatge de nutrients i l'atenuació d'inundacions (Borja et al. 2012a). Ibáñez et al. (1997) va exposar que en els estuaris amb falca salina (com ara l'Ebre), el règim de falca s'estableix durant períodes de cabals baixos del riu (per sota de la mitjana anual), mentre que durant períodes de cabals alts, la falca salina desapareix i l'estuari es converteix en un riu. En altres tipus d'estuaris altament estratificats (com ara els fiords), el règim de falca salina s'estableix durant els períodes de cabals alts, mentre que amb cabals baixos, aquests ecosistemes es converteixen en estuaris parcialment barrejats. El particular règim hidrològic dels estuaris amb falca salina té conseqüències ecològiques importants, ja que les fluctuacions de salinitat i altres factors fisicoquímics són més accentuades, irregulars e impredictibles que en altres tipus d'estuaris, reduint la productivitat i la diversitat en les comunitats biològiques.

En les últimes dècades, les activitats humanes a les conques i costes han empobrit la qualitat dels rius i estuaris. Les pressions antròpiques inclouen, entre d'altres, la regulació del

cicle hidrològic a través d'embassaments, la sobreexplotació dels recursos hídrics, la reducció de les aportacions de sediments, la contaminació química i la proliferació d'espècies exòtiques. Un dels impactes antropogènics més estudiats és l'increment de les concentracions de nutrients i contaminants en els ecosistemes fluvials, els quals es poden acabar acumulant en els estuaris i zones adjacents. Com a conseqüència, molts estuaris han passat d'un estat mesotròfic a un estat eutròfic (Ketchum 1969). Els efectes de l'eutrofització inclouen un augment dels "blooms" de fitoplàncton (alguns formats per espècies tòxiques), la reducció de la transparència de l'aigua, canvis en la composició i distribució de les macroalgues i plantes vasculars, un pH elevat i un esgotament de l'oxigen dissolt (Smith et al. 1999). En el cas dels estuaris amb falca salina, els efectes de l'eutrofització són especialment dràstics, ja que es poden desenvolupar condicions d'anòxia estricta a la falca, la qual cosa dificultarà l'establiment d'una comunitat biològica (Ibáñez et al. 1995).

Per tal de protegir i millorar la qualitat dels ecosistemes aquàtics, la Unió Europea va desenvolupar la Directiva Marc de l'Aigua (DMA, Unió Europea 2000), en la qual els mètodes tradicionals utilitzats per a l'avaluació de la qualitat de l'aigua (p. ex., la mesura directa de contaminants) es van substituir per un enfoc integral amb l'objectiu d'avaluar "l'estat ecològic" de les masses d'aigua. La finalitat d'aquesta directiva és aconseguir un estat ecològic de les masses d'aigua europees "bo" abans del 2015 (els estuaris s'inclouen dins de la categoria d'aigües de transició). L'estat ecològic d'una massa d'aigua es defineix com "una expressió de la qualitat de l'estructura i el funcionament dels ecosistemes aquàtics associats a les aigües superficials" (article 2, incís 21). Dit d'una altra manera, l'avaluació de la qualitat ecològica dels ecosistemes aquàtics segons la DMA ha de descriure la relació entre els organismes i les múltiples pressions ambientals, incloent els elements hidrològics, morfològics i fisicoquímics (Allan et al. 2006; Borja et al. 2004; Hering et al. 2006; Logan i Furse 2002). La directiva defineix cinc classes d'estat ecològic d'acord a la desviació que presenten comparant-los amb les condicions de referència (molt bo, bo, mediocre, deficient i dolent), i s'utilitzen diversos "elements de qualitat biològics" com a bioindicadors (peixos, macroinvertebrats i flora aquàtica). L'avaluació de l'estat ecològic mitjançant bioindicadors inclou la composició taxonòmica, diversitat, riquesa, densitat d'espècies i diversos índexs que comparen proporcions d'espècies sensibles i tolerants a la contaminació (Borja et al. 2009; Hering et al. 2006).

La implementació de la DMA a les aigües de transició (i més específicament als estuaris) no és senzilla i hi ha molta menys informació comparada amb l'existent per als ecosistemes d'aigua dolça. En primer lloc, la variabilitat hidrològica i fisicoquímica de les aigües de transició obliga a dividir i avaluar les masses d'aigua per separat d'acord amb gradients ambientals, com per exemple la salinitat (Borja et al. 2004), no només entre masses d'aigua, sinó també dins d'un únic sistema estuarià. En segon lloc, ja que la majoria d'estuaris han estat sota la influència humana durant segles, les condicions de referència (és a dir, sense o sota molt poca pertorbació humana) són inexistents i només es poden identificar a través de dades històriques i paleontològiques (Kauppila et al. 2005), models matemàtics (Muxika et al. 2007) o segons

l'opinió d'experts (Bald et al. 2005). Finalment, tot i que els estuaris estan exposats a un alt grau d'impactes antropogènics, en general són ecosistemes altament dinàmics i rics en nutrients i matèria orgànica de per sí (Dauvin i Ruellet 2009). Per tant, les comunitats biològiques que habiten en els estuaris presentaran característiques similars (p. ex. en la composició, diversitat i distribució d'espècies) a les comunitats biològiques que habiten en ecosistemes degradats, la qual cosa fa que sigui molt difícil distingir entre els efectes de les pressions naturals i antropogèniques (Dauvin 2007; Elliott i Quintino 2007). Aquesta dificultat es troba descrita com a "Estuarine Quality Paradox" i ha estat el focus d'estudis recents dedicats a l'avaluació de l'estat ecològic dels estuaris (p. ex. Borja et al. 2012b; Ellis i Bell 2013; Rakocinski 2012; Veríssimo et al. 2013).

L'ecologia i la taxonomia de les diatomees bentòniques als estuaris

Les diatomees (Bacil·lariòfits) són el grup més divers d'algues eucariotes, amb un nombre estimat d'espècies de l'ordre dels 10^4 - 10^5 (Mann i Droop 1996). Poden viure a tot tipus d'hàbitats (terrestres, d'aigua dolça i marins), i són sovint el component més important de les comunitats d'algues bentòniques i planctòniques d'aquests hàbitats (Smol i Stoermer 2010). Les diatomees es caracteritzen per la presència d'una paret cel·lular silícia, formada per dues valves que constitueixen el frústul. La mida i la forma del frústul són caràcters diagnòstics utilitzats per a la identificació d'espècies (Smol i Stoermer 2010).

Les diatomees bentòniques són particularment importants en els sistemes estuarians, ja que constitueixen un dels components principals dels biofilms (p. ex. Méléder et al. 2007), contribueixen fins al 50% del total de la producció primària dels estuaris (Underwood i Kromkamp 1999), tenen una funció essencial en la cadena alimentària (Lamberti 1996), estan involucrades en diversos cicles biogeoquímics, com per exemple el del nitrogen i el de la sílice (Thornton et al. 2002) i ajuden a estabilitzar els sediments (Underwood i Paterson 1993). Tot i la recerca significativa sobre l'ecologia de les diatomees bentòniques estuarianes i el seu paper en el funcionament dels biofilms (revisat per Trobajo i Sullivan 2010), no és senzill determinar els factors que afecten a la composició i distribució de les espècies en el camp, ja que:

i. La covariació fisicoquímica inherent en els estuaris complica la identificació dels paràmetres ambientals que afecten directament a les comunitats de diatomees bentòniques (Underwood et al. 1998). Les preferències ecològiques de les diatomees d'aigua dolça es poden inferir a partir dels patrons de distribució al camp (p. ex. van Dam et al. 1994), però en el cas de les diatomees estuarianes es necessiten estudis experimentals per a facilitar la interpretació de les correlacions ambientals, i en aquest sentit s'han fet relativament poques investigacions (amb algunes excepcions importants, p. ex. Admiraal 1976, 1977, 1984; Admiraal i Peletier 1979, 1980; Admiraal et al. 1982; Smith i Underwood 2000; Trobajo et al. 2004; Underwood et al.1998; Underwood i Provot 2000; aquesta tesi).

ii. La majoria d'espècies de diatomees abundants als estuaris tenen una taxonomia difícil, en part perquè són de mida petita i estructura delicada (5 - 30 µm) i en part perquè tenen pocs caràcters morfològics distintius, els quals poden ser difícils d'observar. A més a més, els canvis morfològics i morfomètrics que ocorren durant el cicle de vida de les diatomees (p. ex. Amato et al. 2005; Armbrust i Chisholm 1992; Trobajo et al. 2006) o com a resposta a les condicions ambientals (p. ex Håkansson i Chepurnov 1999; Trobajo et al. 2004; Wood et al. 1987) suposen una dificultat addicional a la seva taxonomia. Com a conseqüència, la documentació bibliogràfica de les espècies de diatomees que habiten en estuaris és sovint pobre i confusa.

iii. Al no existir cap flora detallada i específica per a identificar diatomees estuarianes, la determinació de les espècies que habiten en aquests ambients requereix de consultes a treballs aïllats, dispersos i a vegades de difícil accés. Per tant, les espècies de diatomees estuarianes s'identifiquen utilitzant bibliografia taxonòmica dissenyada per al seu ús en ecosistemes d'aigua dolça, i en molts casos s'aplica el criteri de "la morfologia més semblant". Com a resultat, moltes espècies estuarianes són identificades com a espècies originalment descrites per a sistemes d'aigua dolça, sense una clara evidència de que es tracti de la mateixa espècie. Tot i això, no es coneix l'ecologia en ambients salobres i/o marins de la majoria d'espècies descrites a partir d'ecosistemes d'aigua dolça.

Qualsevol estudi ecològic pressuposa el previ coneixement taxonòmic de les espècies que habiten l'ecosistema. Tot i que el propòsit d'aquesta tesi no és estrictament taxonòmic, les dificultats anteriorment esmentades ens alerta de la importància i l'especial atenció que requereix la taxonomia de les diatomees estuarianes. Recentment s'han aplicat nous enfocs utilitzant tècniques moleculars i experiments de reproducció sexual que han demostrat que, tot i que essencial, la identificació d'espècies basada en la morfologia del frústul pot ser insuficient, i que per tant, la diversitat de les diatomees ha estat probablement subestimada (p. ex. Mann 2010). Alguns complexos d'espècies amb morfologies molt similars o idèntiques contenen dos o més grups genèticament i/o reproductivament diferents (espècies críptiques o pseudocríptiques: p. ex. Amato et al. 2007; Beszteri et al. 2005; Créach et al. 2006; Evans et al. 2007; Kooistra et al. 2010; Poulíčková et al. 2010; Trobajo et al. 2009; Vanormelingen et al. 2008). Algunes d'aquestes espècies críptiques i pseudocríptiques també poden presentar diferents preferències ecològiques, geogràfiques i d'hàbitat (p. ex. Casteleyn et al. 2008; Degerlund et al. 2012; Kaeriyama et al. 2011; Kooistra et al. 2008; Poulíčková et al. 2008; Orsini et al. 2004; Souffreau et al. 2012; Vanelslander et al. 2009). El descobriment d'una diversitat críptica suposa un nou repte per a l'ecologia de les diatomees estuarianes, ja que l'ampli rang de tolerància a les fluctuacions ambientals que s'ha documentat per a la majoria d'aquestes espècies podria ser un artefacte en part provocat per l'agrupament d'espècies morfològicament molt similars però amb preferències ecològiques diferents.

L'ús de les diatomees bentòniques com a indicadors biològics als estuaris

Els macròfits i el fitobentos són part de la flora aquàtica considerada per la DMA com a elements biològics de qualitat per als ecosistemes d'aigua dolça (Annex V), i les diatomees són considerades representatives del fitobentos (Kelly et al. 2008). La seva ubiqüitat, la seva resposta directa, ràpida i sensible als canvis fisicoquímics i la seva conservació en els sediments durant períodes llargs els fa bons indicadors de qualitat de l'aigua tant per als canvis ambientals presents com del passat (Smol i Stoermer 2010). Com a resultat, els estats membres de la Unió Europea utilitzen habitualment més de 20 índexs basats en diatomees bentòniques per tal d'avaluar l'estat ecològic dels rius i rierols, sent alguns d'ells recentment adaptat per als llacs (Stenger-Kovács et al. 2007).

Encara que en les aigües de transició l'ús del fitobentos no és obligatori segons la DMA (secció 1.2.3 de l'annex V), la importància ecològica d'aquesta comunitat ha contribuït a un gran nombre d'estudis sobre la seva distribució i les funcions ecològiques (revisat a Trobajo i Sullivan 2010). Juntament amb altres comunitats bentòniques (p. ex. els macroinvertebrats), les diatomees poden integrar els efectes de diverses pressions ambientals millor que altres indicadors biològics, com ara els peixos o el fitoplàncton (Andrén i Jarlman 2008; Hering et al. 2006; Stevenson et al. 2010), la qual cosa és especialment útil en ecosistemes altament dinàmics com els estuaris. No obstant, mentre que en el cas dels macroinvertebrats s'han ajustat i/o desenvolupat nous índexs per als ecosistemes estuarians (revisat a Borja i Dauer 2008, Díaz et al. 2004), no existeix cap índex de diatomees específic per a estuaris, i els estudis que han aplicat els índexs existents en aquests ecosistemes són molt escassos (Bauer et al. 2007; Bogaczewicz-Adamczak i Dziengo 2003; Della Bella et al. 2007; Zgrundo i Bogaczewicz-Adamczak 2004).

Context de l'estudi: L'estuari de l'Ebre

La conca de l'Ebre té una gran importància socioeconòmica ja que el riu Ebre és el riu espanyol més gran pel que fa al cabal (11.982 hm^3/any abans d'entrar a la desembocadura, segons www.chebro.es). De fet, és un dels rius més importants de la Mediterrània i té una de les majors conques a Europa (Tockner et al. 2009). D'altra banda, el Delta de l'Ebre és un dels deltes més grans de la Mediterrània nord-occidental (330 km^2) (Curcó et al. 2002). Les activitats humanes en els dos darrers segles han estat molt intenses al riu Ebre i al seu estuari, amb un ús exhaustiu de l'aigua per a la indústria i les principals ciutats, però sobretot per les activitats agrícoles que van provocar una sobreexplotació dels recursos hídrics i alts nivells d'eutrofització fins a mitjans dels anys 90. Des de llavors, la millora en el tractament de les aigües residuals urbanes a les principals ciutats de la conca de l'Ebre i la reducció dels fosfats en els detergents han induït una forta disminució en la concentració de fòsfor amb la conseqüent limitació del fitoplàncton i l'augment de la transparència de l'aigua (Ibáñez et al. 2008, 2012). Aquest procés d'oligotrofització ha produït un canvi en la flora aquàtica, passant d'estar dominada pel fitoplàncton en els anys 90 a estar-ho pels macròfits en l'actualitat, fet que

també ha donat lloc a altres canvis en les comunitats biològiques. A l'estuari de l'Ebre, la principal conseqüència de la disminució de les aportacions de fòsfor ha estat una important disminució del fitoplàncton a la capa superior (aigua dolça) i un augment en la concentració de fitoplàncton a la falca salina (Falco et al. 2006), la qual cosa ha permès revertir les condicions d'anòxia presents durant el període d'eutrofització.

La conca del riu Ebre ha estat fortament modificada per una sèrie d'embassaments (aproximadament 200), la majoria construïts entre 1940 i 1970. A la part baixa del riu (a 100 km de la desembocadura), hi ha dos embassaments principals (Mequinença i Riba-roja), construïts durant la dècada dels 1960 per usos hidroelèctrics i amb una capacitat de 1534 hm^3 i 207 hm^3 respectivament. Les característiques i el funcionament d'aquests embassaments afecten la hidrologia, la geomorfologia i l'ecologia de la part baixa del riu Ebre, amb una alteració del ritme i la magnitud de les crescudes (Batalla et al. 2004), la dinàmica dels sediments (Vericat i Batalla 2006; Batalla i Vericat 2009), la temperatura i les característiques fisicoquímiques de l'aigua (Ibáñez et al. 1995; Prats et al. 2010; Sabater et al. 2008). El cabal mitjà anual era 592 $m^3 s^{-1}$ al començament del segle XX, però ha anat decreixent a partir de la dècada dels 1970 (aprox. 400 $m^3 s^{-1}$ en l'actualitat) a causa del reg (90% de l'aigua que es consum a la conca) i l'evaporació als embassaments (Ibáñez et al. 1996; Ibáñez i Prat 2003). Per tant, l'alteració del règim hidrològic a causa dels embassaments i l'extracció d'aigua sembla ser avui dia la pressió antropogènica més rellevant a la part baixa del riu Ebre, i té com a conseqüència principal una major persistència de la falca salina a l'estuari tant a nivell espacial com temporal.

Aquesta tesi es centra en els últims 40 km del riu Ebre que comprenen l'estuari de l'Ebre (40º 43' 16,59'' N, 0º 40' 37,79'' E). Es considera un estuari de falca salina micromareal i ocupa una àrea d'aproximadament 10 km^2 dins del Delta de l'Ebre. Tant l'estuari com el delta es troben influenciats per un clima Mediterrani, que es caracteritza per hiverns moderats (la temperatura mitjana al gener és de 9.18 ºC) i estius subàrids (temperatura mitjana mensual de 25.68 ºC a l'agost). Les precipitacions (mitjana anual de 548 mm) són molt escasses a l'estiu i es concentren a la tardor, encara que la variabilitat interanual és molt alta. Els forts vents durant febrer-maig i setembre-desembre poden incrementar el rang de fluctuació del nivell de l'aigua des de 0.2 m (l'habitual) fins a > 1 m (Curcó et al. 2002).

L'estuari de l'Ebre és l'estuari Mediterrani amb falca salina més estudiat, encara que la major part de la seva recerca s'ha centrat en el funcionament hidrològic de la falca salina i dinàmica i composició dels nutrients i del fitoplàncton (Casamayor et al. 2001; Falco et al. 2006, 2010; Guillén i Palanques 1992; Ibáñez et al. 1995, 1997, 1999; Pérez et al. 2002, 2009; Sierra et al. 2002, 2004). La major transparència de l'aigua i la conseqüent variació d'un ecosistema eutròfic a un mesotròfic ha millorat les condicions de llum i nutrients a la falca salina, la qual cosa ha permès l'establiment de comunitats bentòniques. No obstant això, malgrat la importància ecològica i socioeconòmica de l'estuari de l'Ebre, els estudis sobre la seva biota bentònica són encara molt escassos i basats només en la comunitat de macroinvertebrats (Ibáñez et al. 1995; Nebra et al. 2011).

Aquesta tesi és el primer estudi sobre la comunitat de diatomees bentòniques de l'estuari de l'Ebre i sorgeix com a conseqüència de la necessitat d'avaluar el seu estat ecològic segons la DMA. Forma part d'un estudi més ampli sobre les característiques de les comunitats bentòniques (és a dir, diatomees i macroinvertebrats) i el seu ús com a bioindicadors a l'estuari de l'Ebre, el qual ha estat financiat per l'Agència Catalana de l'Aigua (ACA). La tesis s'ha enfocat com a un estudi multidisciplinari, on les dades de camp s'han combinat amb estudis taxonòmics, experimentals i moleculars.

Objectius

L'objectiu general de la tesi és avançar en el coneixement de l'ecologia i la taxonomia de les diatomees bentòniques d'un estuari Mediterrani altament estratificat (l'estuari de l'Ebre), per tal d'avaluar el seu ús potencial com a indicadors biològics d'aquest ecosistema. Per a assolir-ho, es va estudiar la composició de les espècies de diatomees bentòniques i es van determinar els factors que afecten l'estructura de la comunitat (**Capítol 1**). Un cop detectades les principals pressions antropogèniques a l'estuari de l'Ebre, la resposta de les diatomees a aquestes pressions i el seu paper com a bioindicadors es va testar mitjançant: i) l'avaluació dels índexs de diatomees existents, i ii) la identificació de grups d'espècies indicadores de condicions ambientals potencialment alterades (**Capítol 2**). Els estudis de camp es van complementar amb estudis experimentals per tal de testar la resposta de les espècies al principal gradient ambiental a l'estuari de l'Ebre (és a dir, la salinitat) (**Capítols 3 i 5**). Es van estudiar detalladament diversos aspectes taxonòmics per tal d'ajudar a la comprensió i interpretació dels resultats ecològics. En primer lloc, es va documentar la variabilitat morfològica i la resposta ecofisiològica de diverses espècies a la salinitat (**Capítol 3**). Seguidament, es va comparar la morfologia de les valves d'algunes espècies petites i morfològicament molt similars del gènere *Nitzschia* (un dels més abundants a l'estuari de l'Ebre) (**Capítol 4**). Finalment, la tesi es va centrar en l'espècie *Nitzschia inconspicua* per tal d'analitzar la variabilitat morfològica, genètica, reproductiva i ecofisiològica d'una espècie taxonòmicament complexa però ecològicament important, tant en ecosistemes d'aigua dolça com en aigües de transició (**Capítol 5**). En cada capítol de la tesis es van plantejar una sèrie de preguntes específiques:

Capítol 1:

> Quina és la composició d'espècies de diatomees bentòniques a l'estuari de l'Ebre? Quins gradients ambientals determinen l'estructura de la comunitat? Existeix algun patró espacial o temporal pel que fa a l'agrupament de les espècies? Hi ha altres factors abiòtics a més dels fisicoquímics que afectin a la composició i distribució de les espècies? (p. ex. substrats naturals versus artificials).

Capítol 2:

> Quines són les principals pressions humanes a l'estuari de l'Ebre? Són les diatomees bentòniques bons bioindicadors de condicions alterades a l'estuari de l'Ebre? Hi ha

algun grup d'espècies que pugui ser utilitzat com a indicador de condicions ambientals potencialment alterades? És factible i fidedigne aplicar els índexs de diatomees existents per tal d'avaluar l'estat ecològic de l'estuari de l'Ebre i/o altres aigües de transició?

Capítol 3:

Quins són els efectes de la salinitat sobre el creixement i la morfologia de la valva d'algunes espècies de diatomees estuarianes? Aquests efectes tenen alguna repercussió a nivell taxonòmic? Tenen totes les espècies estudiades el mateix grau de tolerància als diferents tractaments de salinitat? Hi ha algun patró de variació morfològica i/o de creixement compartit per les espècies en resposta a la salinitat?

Capítol 4:

És possible distingir algunes de les espècies petites i taxonòmicament complexes del gènere *Nitzschia*, freqüents i abundants no només a l'estuari de l'Ebre, sinó també en molts altres ecosistemes? És factible la identificació d'aquestes espècies en futurs estudis de camp? Tenen aquestes espècies diferents respostes ecofisiològiques que facin especialment important la seva correcta identificació de cara als sistemes de monitoratge?

Capítol 5:

Quins són els factors que fan que *N. inconspicua* sigui una espècie taxonòmicament complicada i amb una ecologia confusa, tot i ser abundant en molts ecosistemes aquàtics i tenir, per tant, bones oportunitats per al seu estudi? Hi ha algun patró morfològic entre les diferents poblacions pertanyents a *N. inconspicua*? Hi ha alguna relació entre la variabilitat morfològica, ecofisiològica, reproductiva i molecular d'aquesta espècie? És *N. inconspicua* una sola espècie amb un ampli espectre ecològic o està composta per diverses espècies críptiques, cadascuna amb preferències ecològiques discretes pel que fa a la salinitat?

Discussió general

Aquest apartat té com a objectiu fer una síntesi global dels resultats obtinguts en cada un dels capítols anteriors. Un dels principals resultats de la tesi ha estat el comprovar que la comunitat de diatomees bentòniques pot ser potencialment utilitzada com a indicadora biològica dels principals impactes antropogènics a l'estuari de l'Ebre (i per extensió, possiblement també en altres estuaris amb falca salina). Tot i això, abans d'incorporar les diatomees bentòniques en futures metodologies de monitoratge, s'han de resoldre diversos obstacles en el seu ús per a l'avaluació de l'estat ecològic de l'estuari de l'Ebre, no només com

a resultat de la poca eficàcia dels índexs de diatomees existents, sinó també per l'escàs coneixement ecològic i taxonòmic de les espècies que habiten aquest tipus d'estuaris.

Hidrologia de l'estuari, salinitat i ecologia de les diatomees estuarianes

L'estuari de l'Ebre, així com altres sistemes estuarians prèviament estudiats (revisat a Trobajo i Sullivan 2010), presenta una comunitat ben establerta de diatomees bentòniques, tant a nivell de superfície com de fondària. No obstant això, segons la Directiva Marc de l'Aigua (DMA), ni les diatomees ni altres components del fitobentos són obligatoris com a elements biològics de qualitat per a les aigües de transició i costaneres. L'exclusió del fitobentos en aigües costaneres obertes és raonable, ja que no pot sobreviure a grans profunditats. Però aquest no és el cas de les masses d'aigua de transició interiors i relativament poc fondes, on els sediments de fons es troben prou aprop de la superfície com per a rebre la suficient llum per a la fotosíntesis, com és el cas de l'estuari de l'Ebre.

L'estat ecològic del riu Ebre ha estat avaluat per la Confederació Hidrogràfica de l'Ebre (CHE, http://www.chebro.es/) utilitzant la comunitat de diatomees bentòniques. Els punts de mostreig van abastar la major part de la conca, amb tres punts situats a 30 - 40 km aigües amunt de la desembocadura, els quals inclouen el límit superior de l'estuari de l'Ebre. En aquesta àrea, l'estat ecològic del riu Ebre durant 2007 - 2008 (últim període mostrejat per la CHE) es va classificar com a "moderat" utilitzant els índexos de diatomees IPS i CEE, i "deficient" segons l'índex IBD. Exceptuant els pocs casos esmentats, l'estuari de l'Ebre no ha estat monitoritzat per la CHE, tot i que tant l'estuari com el riu es troben estretament connectats pel que fa al seu estat ecològic i, per tant, les característiques fisicoquímiques i hidrològiques de l'estuari de l'Ebre dependran en gran mesura de la influència dels processos fluvials.

Aquesta tesi ha demostrat que la comunitat de diatomees bentòniques a l'estuari de l'Ebre es troba principalment influenciada per la seva dinàmica hidrològica (és a dir, per la influència marina i per llargs períodes de cabals baixos i estables que determinen la posició i persistència de la falca salina). La hidrologia dels estuaris amb falca salina està determinada per l'equilibri entre el riu i el mar i la interacció de les fluctuacions ambientals associades. En concret, aquesta tesi s'ha centrat principalment en la salinitat, ja que és un dels gradients fisicoquímics clau que caracteritzen els estuaris (Hansen i Rattray 1966) i ha estat prèviament considerada un factor important que afecta a la composició de la comunitat de diatomees bentòniques en altres ecosistemes estuarians (p. ex. Bak et al. 2001; Laird i Edgar 1992; Thornton et al. 2002; Watt 1998). La salinitat afecta al creixement de les espècies de diatomees que habiten a l'estuari de l'Ebre, tot i que el tipus de resposta i les preferències de salinitat són específiques de cada espècie.

Prèviament s'han documentat les diatomees estuarianes com a eurihalines i amb amplis espectres ecològics (Bate i Smailes 2008; Forster et al. 2006; Hassan et al. 2006; Sullivan i Currin 2000; Underwood 1994; Underwood i Kromkamp 1999; Wilderman 1987). Aquests

estudis s'han dut a terme en estuaris mareals amb un marcat gradient oscil·latori de salinitat a causa de l'amplitud i freqüència de les marees i per tant, on el fet de ser eurihalí és un avantatge. Contràriament, la columna d'aigua en els estuaris amb falca salina es pot trobar altament estratificada durant períodes de temps llargs. En aquests tipus d'estuaris, com és l'estuari de l'Ebre, les espècies de diatomees eurihalines són dominants sota condicions fluctuants, com per exemple, en situacions amb fortes variacions de cabal, en les quals la falca salina desapareix (cabals alts) o s'estableix ràpidament (cabals baixos), especialment en punts propers al mar. No obstant això, sota condicions hidrològiques estables (p. ex. quan la columna d'aigua es troba estratificada durant un període llarg degut a cabals baixos, o quan trobem una situació completament fluvial causada per cabals alts), diverses espècies amb un espectre ecològic més restringit poden coexistir amb les espècies eurihalines tant a escala vertical com horitzontal.

La presència d'espècies de diatomees descrites en ambients marins com ara *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina, Gomphonemopsis obscura* i *Parlibellus* cf. *berkeleyi* a l'estuari de l'Ebre serà especialment important ja que es poden considerar indicadores de la presència de falca salina durant llargs períodes de temps a causa d'un cabal persistentment baix. Tot i que aquestes espècies van mostrar un òptim de salinitat alt (> 17 mS/cm) i altes especificitats per a condicions estuarianes (> 96%), es van trobar tant en mostres superficials com de fondària en punts propers al mar, indicant una certa tolerància a les fluctuacions de salinitat. Podem concloure que la majoria de diatomees bentòniques a l'estuari de l'Ebre són tolerants a les fluctuacions ambientals, amb un grau de tolerància específic per l'espècie. Com a resultat, la identificació de grups d'espècies indicadores tindrà més valor a l'hora d'inferir les condicions ambientals de l'estuari que la presència individual de cada espècie per separat.

Tot i que la salinitat pot ser considerada representativa de la influència marina i la dinàmica de la falca salina, les anàlisis multivariants i la incongruència entre les preferències de salinitat inferides del camp i les mesurades al laboratori en algunes espècies suggereixen que la comunitat de diatomees de l'estuari de l'Ebre es veu afectada per múltiples factors tant d'origen natural com antropogènic. La covariació fisicoquímica inherent en estuaris entre la salinitat i altres factors (p. ex. nutrients, oxigen i temperatura) i l'efecte d'altres paràmetres que són difícils d'identificar i mesurar al camp (p. ex. competència, colonització, "grazing") fan molt difícil determinar els efectes per separat de les variables individuals (Cox 1993; Trobajo et al. 2004b; Underwood et al. 1998; Underwood i Provot 2000). D'altra banda, l'efecte similar que tenen els factors d'estrès d'origen natural i antropogènic en les comunitats estuarianes pot comprometre l'avaluació del seu estat ecològic (Elliott i Quintino 2007; Puente i Díaz 2008). En aquest sentit, els estudis experimentals prenen especial rellevància ja que poden ser de gran ajuda a l'hora de desentranyar els factors d'estrès rellevants per a la comunitat de diatomees bentòniques de l'estuari de l'Ebre. En futurs estudis experimentals seria interessant tenir en compte la resposta de les espècies a la salinitat combinada amb altres gradients ambientals

relacionats amb la influència marina i la dinàmica de la falca salina (p. ex. fluctuació de nutrients i de cabals, reducció de la concentració d'oxigen dissolt).

L'ús de diatomees com a indicadors biològics de l'estuari de l'Ebre

Les diatomees bentòniques són potencialment uns bons indicadors biològics de les condicions alterades a l'estuari de l'Ebre ja que en aquest treball s'ha demostrat que són sensibles a la principal pressió antropogènica actual, és a dir, a l'alteració del cabal del riu Ebre i de la dinàmica de la falca salina. S'han descrit diferents grups d'espècies de diatomees indicadores de condicions fluvials (aigua dolça) i de condicions estuarianes (salobres i/o marines). La presència d'espècies d'aquest últim grup, juntament amb les espècies indicadores d'una falca salina establerta a llarg termini es pot utilitzar en futurs estudis com un indicador de pressió antropogènica durant períodes específics, com per exemple a la primavera (on haurien de dominar els cabals alts i per tant, l'absència d'influència marina), o quan es troben més amunt del límit habitual de la falca salina (a 18 km de la desembocadura, prop de l'Illa de Gràcia).

L'ús de diatomees com a indicadors biològics a l'estuari de l'Ebre mostra diversos avantatges metodològics quan es comparen amb altres indicadors biològics considerats per la DMA per a les aigües de transició (Annex V). Per exemple, i d'acord amb estudis previs en altres ecosistemes d'aigua dolça i costaners, la comunitat de diatomees bentòniques en ecosistemes altament dinàmics com l'estuari de l'Ebre es troba més afectada per factors ambientals que pel tipus de substrat (Potapova i Charles 2005; Lane et al. 2003; Snoeijs 1994; Winter i Dutie 2000). Per tant, la comunitat de diatomees que colonitza els substrats artificials es pot considerar representativa de la comunitat natural de l'estuari de l'Ebre, la qual cosa facilita en gran mesura les estratègies de monitoratge. Els còdols són el substrat recomanat per al mostreig de diatomees segons la DMA (Kelly et al. 1998). Malauradament, l'accés a aquest tipus de substrats en estuaris no vadejables (cas de l'Ebre) és molt limitat o impossible, ja que la columna d'aigua és bastant profunda i el seu sediment està compost principalment de sorra (Nebra et al. 2011). L'ús de substrats artificials també minimitza l'efecte de mostrejar diferents tipus de substrats dins de la columna d'aigua alhora que permet controlar les condicions de mostreig i el temps de colonització, factors que afecten a composició del fitobentos en general i de les diatomees en particular i, per tant, als valors de qualitat basats en l'aplicació dels índexs (Anderson 1995, 1999; Biggs 1988; Kelly et al. 1998).

En estuaris estratificats, la circulació per advecció es torna més significativa sota llargs períodes amb una falca salina ben establerta, la qual cosa es reflecteix a nivell superficial en un augment de la concentració de la salinitat a mesura que l'estuari s'apropa a la desembocadura. Quan les aigües superficials han arribat a conductivitats d'aproximadament 3 mS/cm o més, trobem una comunitat de diatomees similar tant a les capes superficials com a les de fondària. Per tant, podem identificar situacions alterades que impliquin un augment dels nivells de salinitat a través de la comunitat de diatomees que colonitza la capa d'aigua superficial. El

mostreig a nivells d'aigua superficials és un avantatge per als procediments de monitoratge en estuaris relativament grans com l'estuari de l'Ebre, on les crescudes i fluctuacions fortes i sobtades del cabal poden dificultar la recuperació dels substrats artificials a les zones profundes.

Dificultats en l'ús de les diatomees bentòniques per avaluar l'estat ecològic de l'estuari de l'Ebre

Si bé amb aquest treball hem mostrat que les diatomees bentòniques són bons indicadors biològics d'alteracions hidrològiques a l'estuari de l'Ebre, l'aplicació dels índexs de diatomees existents per a avaluar l'estat ecològic en estuaris amb falca salina (i similars) no és directe ni recomanable. Això es deu a que la majoria dels índexs de diatomees es van desenvolupar a partir i per a ecosistemes d'aigua dolça del Nord i Centre d'Europa (Kelly et al. 2009). Diverses espècies indicadores de l'estuari de l'Ebre no es troben incloses a les bases de dades d'aquests índexs, especialment entre les espècies indicadores de condicions estuarianes, on > 60% són excloses en la majoria dels casos. De fet, algunes de les espècies trobades durant aquesta recerca eren desconegudes per a la ciència (i una s'ha descrit formalment com a *Planothidium iberense* Rovira i Witkowski), mentre que altres ja havien estan prèviament documentades en ambients salobres i marins, com ara *Achnanthes amoena*, *Amphora polita, Navicula mollis*, *Nitzschia constricta* o *N. prolongata* (Chang 1992; Krammer i Lange-Bertalot 1988, 1991; Levkov 2009; Witkowski et al. 2000).

Els índexs de diatomees existents es van desenvolupar com a fruit de la Directiva sobre el tractament d'aigües residuals urbanes (Comunitat Europea, 1991) i com a tal van ser originalment dissenyats per avaluar la contaminació de nutrients i matèria orgànica de les masses d'aigua. Encara que la DMA va introduir l'avaluació de "l'estat ecològic" com un enfoc integral que no només inclou la qualitat de l'aigua, sinó també l'estructura i funció de l'ecosistema en resposta a les pressions antropogèniques (Kelly 2011), no s'han ajustat o desenvolupat índexs específics per a aquest propòsit. Ja que "l'estat ecològic" inclou la resposta de l'ecosistema a diversos tipus de pressió ambiental, s'ha d'anar amb precaució a l'hora d'aplicar els índexs basats en nutrients en ecosistemes que es troben afectats per altres tipus de contaminació (p. ex. metalls pesants, acidesa e hidrocarburs halogenats) o altres pressions antropogèniques com ara l'alteració hidrològica. A l'estuari de l'Ebre, els valors ecològics resultants de l'aplicació dels índexs existents van estar fortament influenciats pel gradient de salinitat i en canvi es van correlacionar molt poc amb les concentracions de nutrients. Només els índexs tròfics (TDI i TID) van mostrar una correlació negativa consistent amb els nutrients, encara que l'estat ecològic resultant (deficient, dolent) no reflecteix l'estat tròfic de la part baixa del riu Ebre segons la concentració de fòsfor, el qual estaria considerat com a mesotròfic seguint la classificació de rius temperats de Dodds et at. (1998).

Els índexs de diatomees existents assumeixen que un augment de les espècies tolerants a les fluctuacions ambientals estarà lligat a una pertorbació significativa associada als nutrients

com a conseqüència de les activitats humanes. Encara que els resultats d'aquest treball estan d'acord amb el domini d'espècies eutrafèntiques i α o β-mesosapròbies (Trobajo et al. 2004b; Witkowski et al. 2009), els seus patrons de distribució podrien estar també indicant tolerància a altres pertorbacions ambientals, independentment dels nivells de nutrients i/o matèria orgànica. A més a més, una gran abundància d'aquestes espècies no sempre implica una degradació en l'estat ecològic, tal com està exposat en la "Estuarine Quality Paradox" (Dauvin i Ruellet 2009; Elliot i Quintino 2007). A l'estuari de l'Ebre, els resultats suggereixen que el domini d'espècies tolerants a l'estrès ambiental sota condicions estuarianes (i els conseqüents valors baixos en l'avaluació de l'estat ecològic a través de l'aplicació dels índexs) es relaciona principalment amb les fluctuacions de la falca salina, que no són necessàriament condicions alterades. Encara que els nivells de nutrients a l'estuari de l'Ebre segueixen un gradient espacial i temporal, els seus valors actuals relativament reduïts fan que l'alteració hidrològica del cabal del riu Ebre i la persistència de la falca salina constitueixin una pressió molt més forta per a les comunitats de diatomees bentòniques.

Les dades autoecològiques per a la majoria d'espècies de diatomees incloses en els índexs actuals s'han inferit dels ecosistemes d'aigua dolça on viuen aquestes espècies, de manera que no es coneix la seva ecologia sota condicions salobres i/o marines (en el cas que hi puguin viure). Pel que fa a la salinitat, espècies descrites d'ambients "d'aigua dolça" o "marins" poden ser tant eurihalines com tolerar diferents rangs de salinitat o ser estenohalines. Les preferències de salinitat per a diverses espècies de diatomees a l'estuari de l'Ebre no sempre van concordar amb treballs previs en altres ecosistemes similars, ni tampoc entre les nostres dades de camp i els experiments de laboratori. Per exemple, l'òptim de conductivitat per a *N. inconspicua* en els nostres estudis de camp va ser de 4 mS/cm (~ 2.4 ppt a 20°C), mentre que en els experiments de laboratori tots els clons van créixer bé en salinitats de 14 ppt a 35 ppt. En altres estudis, com per exemple en rius d'Estats Units, la seva conductivitat òptima s'ha citat en 0.4 mS/cm (Potapova i Charles 2003) i en 0.9 mS/cm a la part oligohalina de l'estuari del riu de la Plata, a Argentina (Licursi et al. 2010). En bibliografia anterior ha estat descrita com una espècie d'aigües dolces i salobres (p. ex. Van Dam et al. 1994), o com a característica dels trams salobres en altres aigües de transició i costaneres com ara el Mar Bàltic (Ulanova i Snoeijs 2006, Ulanova 2009). La determinació de les preferències ecològiques d'una espècie a partir dels patrons de distribució en un ecosistema concret pot donar resultats molt esbiaixats i que sovint estan totalment lligats a les particulars característiques fisicoquímiques dels ambients considerats. Aquesta tesis ha demostrat que els estudis experimentals en condicions controlades són crucials per a dilucidar els rangs ecològics potencials de les espècies, sent especialment necessaris per a aquelles que es troben incloses en la majoria de índexs. Encara que això pot ser un procés llarg, cal determinar les respostes específiques de l'espècie als gradients ambientals que els índexs biològics pretenen avaluar.

La part baixa del riu Ebre i el seu estuari han estat objecte d'una intensa influència industrial i agrícola durant dècades (Ibáñez et al. 2012). Atès que no existeix cap altre estudi

previ sobre les diatomees bentòniques a l'estuari de l'Ebre, no és possible discernir el nivell de degradació de la comunitat de diatomees actual en comparació amb altres moments. D'altra banda, les característiques geomorfològiques, fisicoquímiques i climàtiques del riu d'Ebre fan que sigui difícil trobar ecosistemes similars que puguin ser considerats com a condicions de referència. Per tant, les futures estratègies de monitoratge a l'estuari de l'Ebre s'haurien de basar en un estudi multidisciplinari que combini la modelització amb estudis de camp i experimentals, amb la finalitat de generar hipòtesis sòlides sobre les condicions i comunitats biològiques de referència i la resposta específica de les diatomees bentòniques als possibles factors d'estrès d'origen antropogènic (Muñoz et al. 2012).

La diversitat oculta de les diatomees i la importància d'un enfoc multidisciplinari

La majoria de les espècies de diatomees de l'estuari de l'Ebre són petites (< 30 µm) i algunes d'elles (p. ex. *E. minima*, *N. inconspicua*, *N. filiformis*, *N. palea* i *N. pusilla*) mostren una variabilitat en els caràcters morfològics clàssics (és a dir, longitud, amplada, densitat d'estries i fíbules) deguda al seu cicle de vida i/o a la salinitat. Bona part d'aquestes espècies han estat confoses en la bibliografia, en molts casos a causa d'una discriminació taxonòmica insuficient. Si una espècie no es pot identificar correctament, les seves preferències ecològiques no es descriuran amb precisió i això afectarà al seu valor indicador i a l'avaluació de l'estat ecològic de l'ecosistema utilitzant índexs basats en les dades autoecològiques de les espècies. Per tant, qualsevol estratègia de monitoratge basada en diatomees es veurà obstaculitzada per la manca d'una taxonomia clara i consistent.

Aquesta tesi ha estudiat *N. inconspicua* des d'una perspectiva multidisciplinària (és a dir, combinant anàlisis morfològics, moleculars, ecofisiològics i reproductius), per tal de refinar la seva taxonomia i les seves preferències ecològiques pel que fa a la salinitat. *N. inconspicua* s'ha identificat erròniament i/o combinat amb *N. frustulum*, i els rangs de mida, amplada, densitat d'estries i fíbules definits per *N. inconspicua* sovint es solapen amb els definits per altres espècies de *Nitzschia* com ara *N. abbreviata*, *N. costei*, *N. epiphytica*, *N. innominata*, *N. invisitata* i *N. soratensis* (p. ex. Hoffmann et al. 2011; Krammer i Lange-Bertalot 1988; Lange-Bertalot 1977, 1993; Lange-Bertalot i Simonsen 1978; Trobajo et al. 2004a, b; aquesta tesis). En els Capítols 1, 2 i 3 d'aquesta tesis i d'acord amb els treballs taxonòmics publicats, es van separar les valves més llargues (15 - 30 µm aprox.) amb àpexs més allargats com a *N. frustulum* i les més curtes (5 - 15 µm aprox.) amb àpexs més arrodonits com a *N. inconspicua*. Tot i això, més endavant en la tesis es va demostrar que cada cultiu monoclonal de *N. inconspicua* pot donar lloc als dos tipus de valva depenent del moment en el cicle vital. La longitud de la valva s'ha descrit prèviament com a un caràcter taxonòmic molt variable. La longitud varia durant el cicle vital de l'espècie com a conseqüència natural del mètode de divisió cel·lular i a la vegada es pot trobar influenciada per les condicions ambientals, p. ex. a través de l'alteració del ritme o el patró de divisió cel·lular (p. ex. Amato et al. 2005; Håkansson i Chepurnov 1999; Potapova i Snoeijs 1997; Trobajo et al. 2004a, 2006), o per la inducció d'una

abrupta reducció de mida (Chepurnov et al. 2004). Aquesta tesis ha conclòs que la longitud no es pot utilitzar com a criteri taxonòmic per a distingir *N. frustulum* i *N. inconspicua*.

En el Capítol 4, l'estudi detallat de la morfologia de la valva va permetre la discriminació entre *N. inconspicua* i espècies morfològicament similars com ara *N. frustulum, N. invisitata* i *N. soratensis*. *N. frustulum* és més ampla (> 3 µm) que *N. inconspicua* (< 3 µm), i per tant les valves identificades com a *N. frustulum* en els Capítols 1, 2 i 3 d'aquesta tesis pertanyen a cèl·lules llargues de *N. inconspicua* després de l'auxo-esporulació. *N. inconspicua* i *N. soratensis* no només es poden diferenciar per la forma del pols i les fíbules així com l'aparença de les estries, sinó que també tenen una relació filogenètica llunyana i diferents respostes a la salinitat, i per tant, en treballs ecològics és justificat l'esforç necessari per a discriminar-les. D'altra banda, en el Capítol 5 es posa de manifest que els caràcters morfològics no són suficients per a identificar la variabilitat genotípica i ecofisiològica existent entre les diferents poblacions de *N. inconspicua*. Amb l'excepció de la longitud màxima observada, cap dels altres caràcters morfològics "clàssics" (és a dir, amplada i densitat d'estries i fíbules) van variar significativament entre poblacions de *N. inconspicua* genèticament i fisiològicament diferents.

N. inconspicua presenta una alta diversitat genètica per a *rbc*L i LSU en comparació amb altres espècies del gènere *Nitzschia* (p. ex. *N. palea*) o *Pseudo-nitzschia* (Trobajo et al. 2009, 2010). Les poblacions de *N. inconspicua* estudiades van mostrar tres respostes diferents a la salinitat, la qual cosa suggereix diferències ecofisiològiques entre elles. A més a més, també vam observar diferències en el patró de restitució de la mida, la majoria amb auxo-esporulació a través d'un procés automíctic anomenat pedogàmia, però en algun cas amb un allargament vegetatiu. Aquesta tesi proposa que l'espectre eurihalí atribuït a *N. inconspicua* podria ser resultat d'agrupar diverses espècies críptiques (no distingibles a nivell morfològic) amb rangs de salinitat discrets. No obstant això, no és senzill aplicar un concepte d'espècie per establir els límits dins de *N. inconspicua*. Mentres que el concepte d'espècie filogenètic mostra que *N. inconspicua* té un origen parafilètic i per tant no es pot considerar com a una única espècie, el concepte d'espècie morfològic ens dóna molt poca evidència sobre els límits intraspecífics, i el concepte biològic d'espècie no es pot aplicar ja que *N. inconspicua* és una espècie automíctica.

La troballa d'una variabilitat ecofisiològica dins de *N. inconspicua* pot ser el primer pas per a refinar els amplis rangs ecològics i els valors indicadors descrits anteriorment per a aquesta espècie (p. ex. Van Dam et al. 1994). Així doncs, és altament recomanable que de cara a estudis futurs s'investigui si aquesta variabilitat s'estén a altres gradients ambientals, com ara nutrients, temperatura o oxigen. El fet que les preferències de salinitat són genotípicament específiques i no estan relacionades amb la morfologia suggereix que la diversitat ecofisiològica dins de *N. inconspicua* només es pot detectar a través de marcadors moleculars (p. ex. DNA "barcodes"), tal com s'ha especificat en altres espècies de diatomees com ara *Chaetoceros socialis* (Degerlund et al. 2012; Huseby et al. 2012) i *Pinnularia borealis* (Souffreau et al. 2012). La diversitat en la resposta a la salinitat dins de *N. inconspicua* és especialment rellevant ja que

no només és una espècie abundant i freqüent tant en el fitoplàncton (Pérez et al. 2009) com en el fitobentos de l'estuari de l'Ebre, sinó que també es troba en la majoria dels ecosistemes aquàtics i està inclosa en la majoria dels índexs de diatomees i funcions de transferència paleoecològiques (Gasse 2002; Hadley et al. 2010; Hassan et al. 2009; Rott et al. 2003; Ryves et al. 2011; Van Dam et al. 1994).

En conclusió, l'enfoc multidisciplinari (combinant dades morfològiques, moleculars, ecofisiològiques i/o reproductives) sembla ser l'única manera d'avaluar adequadament la diversitat de diatomees (a qualsevol nivell), i, per tant, és essencial per entendre el que aquesta diversitat implica en els futurs estudis de monitoratge tant per a l'estuari de l'Ebre com per a la resta d'aigües de transició.

Conclusions

1. A l'estuari de l'Ebre i com a conseqüència de la dinàmica de la falca salina es van observar dues condicions ambientals contraposades; una condició fluvial sense o amb una influència marina molt feble i; una condició estuariana caracteritzada per una forta influència marina a les parts fondes i, en menor escala, a nivell superficial en llocs propers al mar.

2. La comunitat bentònica de diatomees a l'estuari de l'Ebre es troba ben establerta tant a nivell superficial com de fondària, ja sigui sota condicions fluvials com estuarianes. La majoria de les 160 espècies trobades són espècies cosmopolites i àmpliament documentades per a altres ecosistemes, com ara *Cocconeis placentula* var. *euglypta* (present en un 99% de les mostres estudiades), tot i que vàries espècies (p. ex. *N. inconspicua*) tenen una taxonomia confosa i la seva identificació no ha estat senzilla.

3. *Planothidium iberense* va ser descrita com a espècie nova per a la ciència, i es pot considerar indicadora d'una condició ecològica particular a l'estuari de l'Ebre.

4. La dinàmica hidrològica de l'estuari de l'Ebre és el principal factor que afecta a la comunitat de diatomees bentòniques tant a nivell espacial com temporal. La influència marina va ser el principal factor que va afectar a la distribució i composició d'espècies a nivell espacial. La variabilitat temporal en la comunitat es va associar amb les fluctuacions del cabal de la part baixa del riu Ebre i cabals baixos durant períodes llargs de temps.

5. La resposta de la comunitat bentònica de diatomees a la dinàmica hidrològica de l'estuari de l'Ebre va ser més rellevant que l'efecte de la salinitat, el tipus de substrat o la concentració de nutrients per separat. Cal tenir en compte que tant la influència marina com el règim hidrològic del riu Ebre són processos ambientals complexes, els quals comprenen la interacció de diversos factors ambientals, i que els efectes

d'aquests factors per separat no es poden determinar només a partir d'estudis de camp.

6. Tot i que la majoria de les espècies de diatomees bentòniques a l'estuari de l'Ebre són tolerants a les fluctuacions ambientals de l'ecosistema, es van identificar associacions d'espècies indicadores de condicions fluvials i estuarianes. A més a més, grups d'espècies amb un baix valor indicador però presents només sota condicions específiques van resultar útils a l'hora d'identificar situacions de prevalença de la falca salina durant un llarg període de temps.

7. *Achnanthidium minutissimum*, *Amphora pediculus*, *A.* cf. *vetula*, *Cocconeis placentula* var. *euglypta*, *C. placentula* var. *trilineata*, *Navicula antonii*, *N. cryptotenella*, *N.* cf. *cryptotenelloides* i *Nitzschia amphibia* són espècies indicadores de condicions fluvials.

8. *Amphora polita*, *Navicula gregaria*, *N.* aff. *mollis*, *N. perminuta*, *N. recens*, *Nitzschia constricta*, *N. inconspicua* (referida com a *N. frustulum*) i *Tabularia fasciculata* són espècies indicadores de les condicions estuarianes.

9. *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *Gomphonemopsis obscura*, *Parlibellus* cf. *berkeleyi* i *Planothidium iberense* són espècies indicadores de la presència de falca salina ben establerta durant un període llarg de temps.

10. Actualment, la principal pressió d'origen humà a l'estuari de l'Ebre és la major presència i estabilitat de la falca salina com a resultat de la forta regulació del cabal, causada per les activitats agrícoles i pel funcionament dels embassaments des dels anys 1960.

11. La comunitat bentònica de diatomees és un bon indicador de les pressions antropogèniques a l'estuari de l'Ebre, ja que es poden detectar condicions hidrològiques alterades a través de grups d'espècies de diatomees indicadores de condicions estuarianes i de presència de falca salina durant un llarg període de temps.

12. Els índexs de diatomees existents van fallar en l'avaluació de l'estat ecològic de l'estuari de l'Ebre, ja que no es van correlacionar adequadament amb els nutrients i moltes de les espècies indicadores de condicions estuarianes no es troben incloses en les seves bases de dades. Per tant, la seva eficàcia en estuaris amb falca salina i altres aigües de transició s'ha de testar abans del seu ús.

13. La salinitat va afectar el creixement i la morfologia de la valva de les espècies estudiades; *Eolimna subminuscula, Nitzschia filiformis* var. *conferta, N. inconspicua* (referida com a *N. frustulum*)*, N. palea* i *N. pusilla*, tot i que la resposta va ser diferent per a cada espècie.

14. L'efecte de la salinitat en la morfologia de les cinc espècies estudiades va ser petit, i per tant, es manté la utilitat d'alguns caràcters taxonòmics clàssics (amplada i densitat d'estries) a l'hora de discriminar aquestes espècies en ambients amb fortes fluctuacions de salinitat.

15. Quatre de les cinc espècies estudiades van créixer bé o moderadament bé en tots els tractaments de salinitat, la qual cosa està d'acord amb l'àmplia distribució que aquestes espècies tenen a l'estuari de l'Ebre. Tot i això, les espècies aïllades d'ambients salobres (*N. inconspicua* i *N. pusilla*) van créixer millor sota condicions de baixa salinitat que no pas les espècies aïllades d'ambients d'aigua dolça (*N. filiformis* var. *conferta* i *N. palea*) sota salinitats altes.

16. *N. frustulum, N. inconspicua, N. soratensis* i *N.invisitata* són espècies taxonòmicament independents, i si s'examinen amb detall, es poden distingir sota el microscopi òptic. La principal diferència entre *N. inconspicua* i *N. frustulum* és l'amplada (promitg de < 3 µm a *N. inconspicua* i > 3 µm a *N. frustulum*). *N. invisitata* i *N. soratensis* es distingeixen de *N. inconspicua* no només per una ultraestructura diferent, sinó també per tenir les fíbules eixamplades a la base. A més a més, *N. invisitata* té les arèoles més marcades, una densitat d'estries lleugerament més baixa i les fíbules al centre de la valva generalment més separades entre sí, mentres que *N. soratensis* té els pols més arrodonits i les estries menys visibles. Finalment, *N. soratensis* i *N. inconspicua* tenen una relació filogenètica llunyana tant per a *rbc*L com LSU, i mentre que la primera va créixer només en condicions estrictes d'aigua dolça, la darrera va créixer en un rang ampli de salinitat (des de condicions d'aigua dolça fins a salinitats de 35 ppt).

17. *N. frustulum* var. *subsalina* i *N. boliviana* són sinònims de *N. inconspicua*. Les tres espècies comparteixen els mateixos trets ultraestructurals i un solapament de les mètriques observades, i per tant no es poden diferenciar morfològicament.

18. *N. inconspicua* té un origen parafilètic incloent altres espècies del gènere *Nitzschia* i *Denticula*, i va mostrar una alta diversitat genètica tant per *rbc*L com per LSU, amb l'agrupament de diversos genotips en tres grups. A més a més, poblacions amb diferents genotips van mostrar diferències en el patró de restitució de mida i en el creixement com a resposta als diferents tractaments de salinitat.

19. La variabilitat ecofisiològica dins de *N. inconspicua* només es pot distingir a través de tècniques d'anàlisi molecular, com ara el "DNA barcoding". Tot i les diferències estadísticament significatives pel que fa a la morfologia de la valva entre poblacions, no es va trobar un patró morfològic que ens permeti identificar les diferents respostes a la salinitat observades experimentalment.

20. La variació ecofisiològica i genètica entre poblacions de *N. inconspicua* sembla indicar que l'àmplia tolerància a fluctuacions ambientals atribuïda a l'espècie podria ser el

resultat d'agrupar diferents espècies morfològicament indistingibles però amb ecofisiologies discretes (almenys per la salinitat).

Bibliografia

Per a la llista de les referències citades consulteu la bibliografia llistada als apartats en anglès de General introduction i General discussion.

Resum de les publicacions

Article 1: Efectes de la dinàmica hidrològica sobre l'estructura de la comunitat bentònica de diatomees en un estuari altament estratificat: el cas de l'estuari de l'Ebre (Catalunya, Espanya). Estuarine, Coastal and Shelf Science 101, 1-14.

L'estudi investiga la distribució de la comunitat de diatomees bentòniques i la seva relació amb els factors ambientals en un estuari Mediterrani altament estratificat, és a dir, l'estuari de l'Ebre. Com a resultat, es mostra la importància de la dinàmica hidrològica a l'hora d'explicar la composició i distribució de la comunitat de diatomees en aquest estuari, en el qual la magnitud i les fluctuacions del cabal del riu impliquen una forta variabilitat fisicoquímica (especialment en aquells punts propers al mar). Durant 2007 i 2008 es van mostrejar 8 punts al llarg de l'estuari tant a nivell de superfície com de fondària, per tal d'englobar tant els gradients fisicoquímics com hidrològics a nivell vertical i horitzontal. L'Anàlisi de Variables Canòniques (CVA) i l'Anàlisi Jeràrquic de Clúster van segregar la comunitat de diatomees en dos grups d'acord amb la dinàmica de la falca salina. El grup d'espècies de condicions fluvials (sense influència de la falca salina) es va caracteritzar per abundàncies altes de *Cocconeis placentula* var. *euglypta* i *Amphora pediculus*, mentre que altes abundàncies de *Nitzschia frustulum* i *Nitzschia inconspicua* van ser característiques de condicions estuarianes (sota la influència de la falca salina). L'Anàlisi de Redundàncies (RDA) va mostrar la resposta estacional dels dos grups d'espècies als cabals del riu Ebre, especialment destacat en el cas de les condicions estuarianes, on les condicions fluctuants van afectar a la comunitat de diatomees tant a nivell espacial com temporal.

Paraules clau: estuari amb falca salina, fitobentos, diatomees, hidrologia, conductivitat, Ebre.

Article 2: L'ús de les diatomees com a indicadors ecològics en estuaris altament estratificats i avaluació dels índexs de diatomees existents. Marine Pollution Bulletin 64, 500–511.

Els índexs de diatomees s'utilitzen per a avaluar l'estat ecològic de rius però els estudis sobre la seva aplicació en estuaris són escassos. En aquest estudi es van identificar les espècies de diatomees indicadores dels principals gradients ambientals i les pressions en un estuari altament estratificat (estuari de l'Ebre); així com també es va avaluar l'aplicabilitat dels índexs de diatomees existents per a aigua dolça. La influència marina com a resultat de la intrusió de la falca salina i de la barreja amb l'aigua del mar va aparèixer com el principal factor que afecta la comunitat de diatomees bentòniques. Es van identificar 3 grups d'espècies: indicadores de condicions fluvials (sense influència marina), indicadores de condicions estuarianes (condicions heterogènies amb conductivitats més altes degut a la influència marina) i indicadores específiques de situacions de falca salina ben establerta. Actualment, la principal pressió

d'origen antropogènic que afecta a la comunitat de diatomees bentòniques de l'estuari de l'Ebre es l'alteració hidrològica com a conseqüència de la regulació i abstracció del cabal del riu Ebre. En l'aplicació dels índexs de diatomees a l'estuari de l'Ebre es van trobar vàries i important limitacions, com ara una resposta inversa amb els nutrients; espècies ecològicament importants no considerades, etc. Per tant, el seu ús en estuaris no és directe ni recomanable.

Paraules clau: índexs de diatomees, estuari estratificat, estat ecològic, espècies indicadores, falca salina, aigües de transició.

Article 3: L'efecte de la salinitat en el creixement i la morfologia de la valva de cinc diatomees estuarianes. Phycological Research 59, 83–90

Es van investigar els efectes de la salinitat en el creixement i en la morfologia de la valva de cinc diatomees bentòniques estuarianes (*Nitzschia pusilla*, *N. frustulum*, *N. palea*, *N. filiformis* var. *conferta* i *Eolimna subminuscula*), aïllades tant d'hàbitats d'aigua dolça com salobres i/o marins. Els 4 cultius de *Nitzschia* van créixer bé en un rang de salinitat ampli, tot i que alguns (*N. pusilla* i *N. frustulum*) van mostrar una tolerància més àmplia (des de medis completament salins fins a salinitats de 9.5 ppt) que altres (*N. palea* i *N. filiformis* var. *conferta* van reduir el seu creixement en salinitats iguals o superiors a 16 ppt). La salinitat va afectar significativament la morfologia de la valva dels 5 clons estudiats, però no es va trobar un patró consistent ni en els caràcters morfològics afectats ni en la direcció dels efectes. Tot i ser significatius, els efectes de la salinitat en la morfologia de la valva van ser molt petits, i per tant, es pot concloure que la utilitat taxonòmica dels caràcters morfològics clàssics no es veu minvada pels canvis de salinitat a l'ambient.

Paraules clau: cultiu, diatomees estuarianes, variabilitat morfològica, *Nitzschia*, plasticitat fenotípica, salinitat.

Article 4: Morfologia i identitat de vàries espècies de diatomees petites i ecològicament importants pertanyents al gènere *Nitzschia*. Diatom Research 28 (1), 37-59

En aquest estudi s'ha estudiat detalladament la taxonomia de diverses espècies petites i ecològicament rellevants pertanyents al gènere *Nitzschia*, les quals es confonen freqüentment amb altres i/o els seus noms han estat erròniament utilitzats. Examinant tant el material tipus com les mostres modernes sota microscòpia òptica i electrònica, es va concloure que *N. frustulum*, *N. inconspicua*, *N. soratensis* i *N. invisitata* són espècies independents. *N. frustulum* s'assembla a *N. inconspicua* en tots els caràcters morfològics examinats, però les valves són més amples i consistentment més llargues. No es va trobar una base morfològica per a separar *N. frustulum* var. *subsalina* o *N. boliviana* de *N. inconspicua* i, per tant, es poden considerar sinònims de *N. inconspicua*. *Nitzschia soratensis*, descrita recentment a Bolívia, ha estat erròniament identificada a Europa, ja sigui sota el nom de *N. inconspicua* (de la qual es

distingeix principalment pels àpexs més arrodonits, les estries del canal rafidi formades per triplets de porus i les fíbules eixamplades a la base quan s'observen sota el microscopi òptic) o com a *N. abbreviata* (de la qual es diferencia per la ultraestructura dels porus).

Paraules clau: diatomees, frústul, ultrastructura, *Nitzschia*, *Nitzschia frustulum*, *Nitzschia inconspicua*, *Nitzschia invisitata*, *Nitzschia soratensis*, taxonomia, material tipus.

Article 5: Diversitat genètica i funcional en la diatomea *Nitzschia inconspicua*. Journal of Phycology (sota revisió)

Nitzschia inconspicua és una espècie ecològicament important, la qual es troba àmpliament distribuïda, habita una gran varietat d'hàbitats tant d'aigua dolça com salobres, i a més a més pot tolerar fortes variacions de salinitat i alts nivells de contaminació orgànica i de nutrients. Tot i això, la seva identificació no és senzilla, ja que el seu frústul té una mida petita i molt pocs caràcters morfològics vàlids per a la diagnosi. A més a més, no hi ha informació sobre la variabilitat genètica i ecofisiològica de l'espècie. En aquest estudi s´han utilitzat dades morfològiques, moleculars (*rbc*L i LSU D1/D3), ecofisiològiques (preferències de salinitat) i reproductives per a investigar si *N. inconspicua* constitueix una sola espècie amb un ampli rang de toleràncies ecològiques o dues o més espècies críptiques amb preferències ecològiques compartides o diferents. Les dades moleculars per a clons aïllats de punts aigües amunt i deltaics dins de la conca de l'Ebre (Espanya) van revelar set genotips dins de *N. inconspicua*, englobats en tres grups. Dos dels grups també van incloure altres espècies de diatomees morfològicament diferents i ben establertes pertanyents als gèneres *Nitzschia* i *Denticula*, esdevenint *N. inconspicua* una espècie parafilètica i amb necessitat de revisió taxonòmica. La majoria dels genotips van resultar ser automíctics, exhibint pedogàmia, i per tant el concepte d'espècies biològic no es pot utilitzar per a establir els límits intraespecífics dins de *N. inconspicua*. Tot i que es van trobar diferències morfològiques entre els clons, aquestes no van ser consistents entre els genotips pertanyents a diferents grups, els quals només es poden definir utilitzant les dades moleculars. Tot i això, la separació e identificació dels genotips de *N. inconspicua* podria ser important des d'un punt de vista ecològic, ja que es van observar tres respostes diferents a la salinitat, fet que ressalta el valor de combinar els estudis morfològics amb el DNA barcoding a l'hora de desenvolupar estratègies de monitoratge en un futur.

Paraules clau: espècies críptiques, diatomees, ecofisiologia, diversitat genètica, LSU rDNA, filogènia molecular, *Nitzschia*, *Nitzschia inconspicua*, espècies parafilètiques, *rbc*L, resposta a la salinitat.

Article 6 (Apèndix): La comunitat de diatomees bentòniques en un estuari Mediterrani amb falca salina: l'estuari de l'Ebre (NE Península Ibèrica. Acta Botanica Croatica 68 (2), 285–300.

El cabal del riu Ebre és el principal factor que controla la dinàmica hidrològica de l'estuari de l'Ebre, essent aquest últim un estuari micromareal i amb falca salina. L'objectiu d'aquest estudi era descriure la composició d'espècies en la comunitat perifítica de diatomees, així com elucidar els principals factors ambientals que afecten a la comunitat. Les mostres de diatomees perifítiques es van recollir en 8 punts al llarg de l'estuari durant l'Octubre de 2007 i el Gener de 2008. La comunitat de diatomees es va mostrejar tant per al substrat natural com per a l'artificial. La fondària, la velocitat de l'aigua, el pH, l'oxigen dissolt, la temperatura, la conductivitat, la clorofil·la total i la clorofil·la *a* de l'aigua, la clorofil·la total perifítica i els nutrients dissolts van ser mesurats a cada campanya. Es van identificar més de 120 espècies de diatomees. Els gèneres més abundants van ser *Cocconeis, Amphora, Navicula* i *Tabularia.* La variabilitat de la comunitat de diatomees es va analitzar mitjançant mètodes multivariants. L'estratificació de la columna d'aigua va afectar a la comunitat de diatomees tant a nivell horitzontal com vertical, incloent un gradient de salinitat, oxigen dissolt i concentració de nutrients.

Paraules clau: diatomea, estuari, falca salina, perífiton, distribució, composició taxonòmica, Ebre, Mediterrani, Espanya.

Article 7 (Apèndix): *Planothidium iberense* sp. nov., una nova espècie de diatomea salobre de l'estuari de l'Ebre, NE Espanya. Diatom Research 26 (1), 99-107.

En aquest estudi es descriu per primer cop la diatomea *Planothidium iberense* sp. nov, trobada en un rang ampli de salinitat a l'estuari de l'Ebre (NE Espanya). La descripció detallada s'ha basat en observacions utilitzant tècniques de microscòpia òptica i electrònica d'escaneig. La nova espècie s'ha comparat amb altres espècies del gènere *Planothidium* morfològicament similars. *Planothidium iberense* es pot distingir de *P. linkei* principalment per les seves característiques morfomètriques, però també per la presència d'estries multiseriades, en contrast amb les estries biseriades presents en *P. linkei. Planothidium iberense* també es distingeix de *P. delicatulum* i *P. septentrionale* per la presència d'una àrea central eixamplada unilateralment a la valva sense rafe, la qual no es troba en les últimes dues espècies esmentades.

Paraules clau: diatomea, Ebre, estuari, Mediterrani, *Planothidium*, taxonomia.

Article 8 (Apèndix): Pedogàmia i auxosporulació en *Nitzschia* secció *Lanceolatae* (Bacillariophyta). Phycologia 52 (2), 204-220.

El procés de pedogàmia (fusió de gàmetes germans produïts dins la mateixa cèl·lula després de la meiosi) ha estat molt poc documentat en diatomees, amb menys de deu exemples confirmats. Un d'aquests, estudiat per L. Geitler, és el cas d'una espècie de diatomea de Illmitz, Neusiedler See (Àustria), identificada com a *Nitzschia frustulum* var. *perpusilla*. En el present estudi s'ha observat l'auxosporulació uniparental en dos cultius del gènere *Nitzschia* aïllats de la part baixa del riu Ebre (Catalunya, Espanya), morfològicament similars al material de Geitler i pertanyents al complex d'espècies de *Nitzschia inconspicua*. A més a més, es va descriure la formació de les auxòspores dels cultius a través de pedogàmia, gràcies a l'observació del nucli a través de la tinció de Feulgen i la documentació de cèl·lules en viu utilitzant microscòpia òptica. Revisant les preparacions microscòpiques originals del material citològic realitzades per Geitler, es van detectar diferències entre el material d'Illmitz i de l'Ebre pel que fa a la llargada de les cèl·lules inicials, l'estructura del *perizonium* i el procés de degeneració del nucli haploide superflu durant la gametogènesi, indicant que les poblacions de l'Ebre i d'Illmitz són genèticament diferents. L'observació de les auxòspores pertanyents a l'Ebre sota microscòpia electrònica d'escaneig va revelar una innovadora estructura longitudinal del *perizonium*, amb asimetria bilateral i una *incunabula* escalada rodejant el zigot encara sense expandir, la qual cosa contrasta amb la *incunabula* estriada d'altres espècies de *Nitzschia* pedogàmiques, com per exemple *Nitzschia fonticola*. La filogènia molecular establerta mitjançant seqüències de *rbc*L i LSU rDNA D1/D3, així com l'avaluació dels arbres filogenètics forçats per tal de considerar les espècies pedogàmiques monofilètiques, sembla indicar que la pedogàmia ha evolucionat independentment almenys dues vegades en el gènere *Nitzschia* secció *Lanceolatae*, una per al llinatge de *N. inconspicua* i l'altra per a *N. fonticola*.

Paraules clau: material citològic arxivat, auxosporulació, citologia, tinció de Feulgen, Incunabula, endogàmia, LSU rDNA, *Nitzschia*, pedogàmia, perizonium, *rbc*L, reproducció sexual.

General introduction

Estuaries: definition, classification and evaluation of its ecological status

Etymologically, the word “estuary” owes its origin to the latin word *aestuarium* which means “land flooded by the tide”. From the 1950s to the present, several definitions and classifications for estuaries have been proposed regarding to their geological, geomorphological, hydrological, chemical and biological characteristics (for an extended review see Ibáñez 1993; Perillo 1995). Here we considered the definition of Ibáñez (1993) since it is general enough to include all estuary types but specific enough to differentiate estuaries from other coastal water bodies:

“*An estuary is a fluvial-marine system (coastal gulf or final part of a river) exposed to a tidal influence and characterised by a variable and not necessarily permanent (at spatial and temporal scale) mixing of marine water and freshwater coming from continental drainage*.”

Hydrological dynamics of estuaries are probably one of the main characteristics used to define them. According to Hansen & Rattray (1966), estuaries can be divided into four types (Fig. 1):

Type 1: The net flow is seaward at all depths and the upstream salt transfer is produced by eddy diffusion. This type includes most well-mixed estuaries but also partially-mixed estuaries (Fig.1a).

Type 2: The net flow is seaward in the surface and reverses at depth. Upstream salt transfer is caused by both advection and eddy diffusion. Although some well-mixed estuaries fall into this category, most type 2 estuaries are partially mixed (Fig. 1b).

Type 3: The net flow is the same as in type 2, but salt transfer is caused mainly by advection. Fjords are included in this type, with a moderate permanent stratification caused by the presence of a deep salt-water layer (Fig. 1c).

Type 4: The vertical stratification is still greater and the advective circulation is very low (Fig. 1d). These highly stratified estuaries are named salt-wedge estuaries and are frequent in seas with low tidal ranges (e.g. Mediterranean and Gulf of Mexico). The salt-wedge position depends mainly on the river flow, being flushed away when the flow is high enough. Some examples of salt-wedge estuaries are the Mississippi, the Rhône and our study site, the Ebro Estuary (Ibáñez et al. 1997).

The presence of the salt wedge divides the water column into an upper freshwater layer from the river and a deep saline layer from the sea, separated by a narrow interphase (Fig. 1d). The strong stratification is not only detectable by a salinity gradient, but also by a different physicochemical composition and ecological functioning in each water layer. While primary

production will be concentrated in the upper layer (which receives most of the light and nutrients from upstream waters), the high water residence and the consumption of organic matter accumulated in the salt wedge can cause strong hypoxic conditions that would favour the increase of: i) reduced compounds (e.g. ammonium) as a result of denitrification by anaerobic bacteria, and ii) orthophosphate due to solubilisation processes (Ibáñez et al. 1995). Hypoxia can be especially significant near the tip of the salt wedge, where water can remain still without renewal for months.

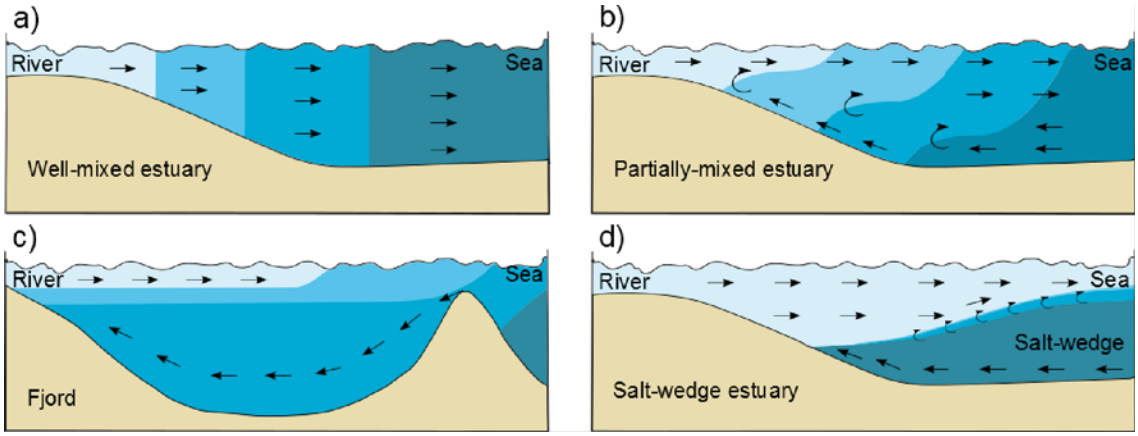

Figure 1. Estuary classification according to their hydrological dynamics. Arrows illustrate the direction of net flow. Different blue colours represent salinity gradient from freshwater (light blue) to salt-water (deep blue). Note the narrow interphase (medium blue) in salt-wedge estuaries (Fig. 1d). Adapted from Arctic Monitoring and Assessment Program (AMAP) webpage (http://www.amap.no).

All estuaries share a high dynamism caused by their tidal range or salt-wedge fluctuation, defining them as naturally stressed ecosystems (Elliott & McLusky 2002; McLusky & Elliott 2007). In the case of salt-wedge estuaries, the unpredictable salt-wedge dynamics will modify physicochemical characteristics of the water column (e.g. from oxygenated and freshwater to marine and anoxic waters) at both spatial and temporal scales (Ibáñez et al. 1995). The complex hydrological functioning of estuaries makes them difficult systems to study and to model conceptually (Trobajo & Sullivan 2010), but at the same time they provide the perfect background to understand the integrated response of organisms to the combination and interaction of different sources of natural and anthropogenic stress.

In general, estuaries are characterised by being highly productive systems that provide a valuable supply of goods and services such as the provision of nursery areas for fish and invertebrate species, feeding and overwintering sites for birds, nutrient recycling and flood attenuation (Borja et al. 2012a). Ibáñez et al. (1997) showed that in strictly salt-wedge estuaries (such as the Ebro), the salt-wedge regime is established during low river flow periods (below mean annual river flow), whereas during high river flows the salt wedge is washed away and the estuary becomes a river. Interestingly, in other types of highly-stratified estuaries (such as fjords), the salt-wedge regime is established during periods of high river flow, whereas during low flows they become partially-mixed estuaries. These contrasting hydrological regimes have

important ecological consequences, since in the case of salt-wedge estuaries, salinity and other physicochemical factors' fluctuations are much stronger, irregular and unpredictable than in other types of estuaries, causing the salt-wedge estuaries to have a lower productivity and diversity in their biological communities.

In recent decades, human activities along river basins and coasts have impoverished the health of rivers and estuaries. Human pressures include, among others, regulation of the hydrological cycle through reservoirs, overexploitation of water resources, reduction of sediment inputs, chemical contamination and the proliferation of exotic species (Fig. 2). Perhaps one of the most studied human impacts is the increase of nutrients and pollutant concentrations in running waters, which can be accumulated in estuaries and adjacent areas. Consequently, most estuaries have changed from a mesotrophic to an eutrophic state (Ketchum 1969). Outcomes from eutrophic conditions include an increase of phytoplankton blooms (some formed by toxic species), water clarity reduction, changes in macroalgal and vascular plant composition and distribution, and elevated pH and an oxygen depletion in the water column (Smith et al. 1999). In the case of salt-wedge estuaries, eutrophication can be especially dramatic since it can lead to strict anoxic conditions in the salt wedge, hampering biological community establishment (Ibáñez et al. 1995).

To protect and improve water quality of aquatic ecosystems, the European Union developed the Water Framework Directive (WFD, European Union, 2000), which replaced traditional water quality methods (e.g. direct measure of pollutants in water) by an integrative approach to evaluate the "ecological status" of water bodies. The aim of this directive is to achieve a "good" ecological status for all European water bodies (estuaries are included under the "transitional waters" category) before 2015. Ecological status is defined as an "expression of the quality of the structure and functioning of aquatic ecosystems associated with surface waters" (Article 2, clause 21). In other words, the assessment of ecological quality under the WFD has to describe the relationship between aquatic organisms and multiple environmental pressures, including the hydrological, morphological and physico-chemical elements (Allan et al. 2006; Borja et al. 2004; Hering et al. 2006; Logan & Furse 2002). Five ecological status classes are defined according to deviation from reference conditions (high, good, moderate, poor and bad) and several "biological quality elements" are used as bioindicators (fish, macroinvertebrates and aquatic flora). The assessment of ecological status using bioindicators include the taxonomic composition, species diversity, richness, density, and several indices comparing ratios of pollution sensitive and tolerant taxa (Borja et al. 2009; Hering et al. 2006).

However, the implementation of WFD in transitional systems (and more specifically in estuaries) is not straightforward and there is much less information compared to freshwater systems. First of all, the high hydrological and physicochemical variability of transitional systems forces their division and separate assessment according to environmental gradients such as salinity (Borja et al. 2004), not only between but also within a single estuarine system.

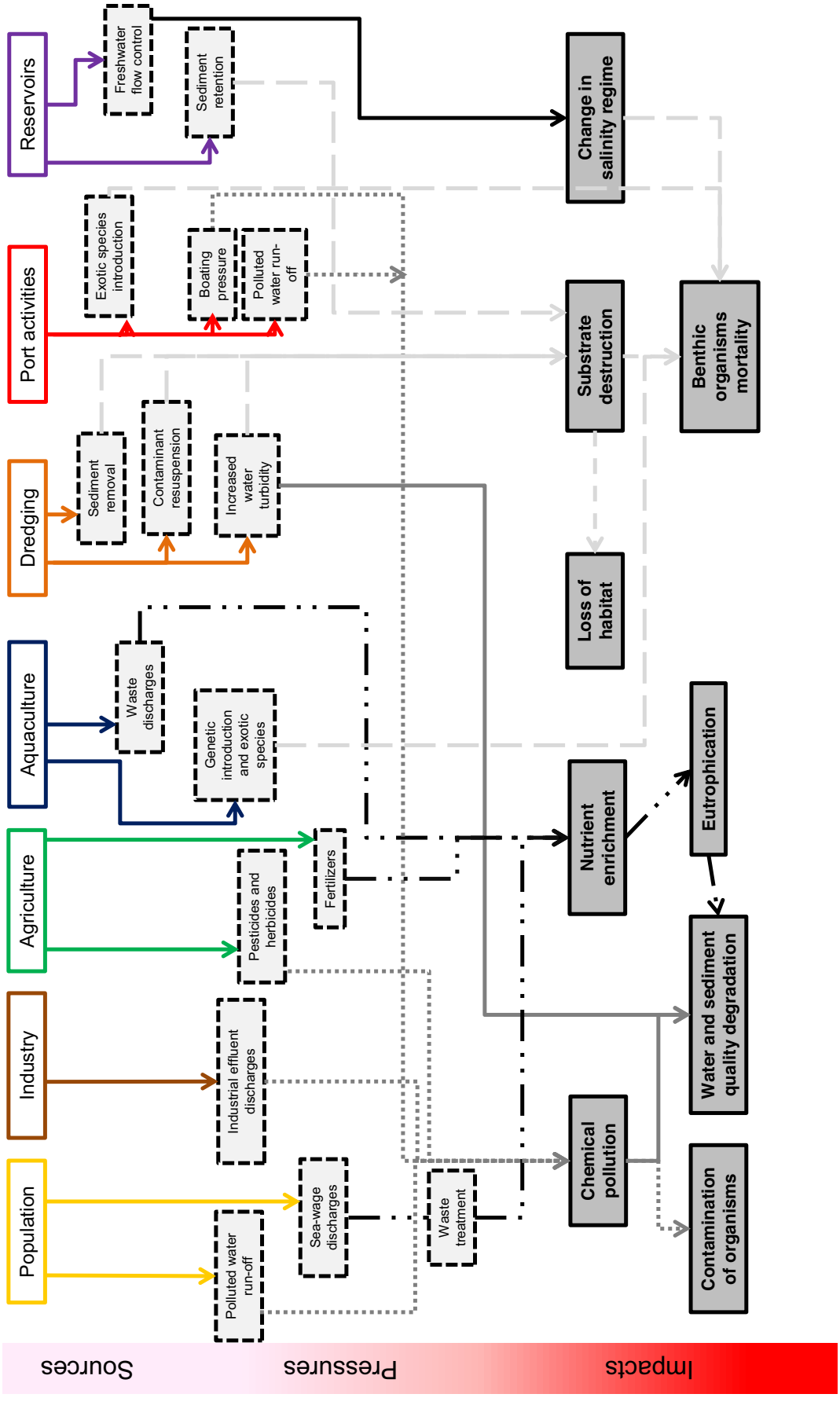

Figure 2. Ecological conceptual model for estuarine pressures and impacts (adapted from Vasconcelos et al. 2007).

Secondly, since most estuaries have been under human influence for centuries, reference conditions (i.e. with no or with minor human disturbance) are non-existent and only identifiable through historical and palaeoecological data (Kauppila et al. 2005), models (Muxika et al. 2007), or expert judgement (Bald et al. 2005). Finally, though estuaries are exposed to a high degree of anthropogenic impacts, they are also naturally stressed, highly dynamic and nutrient-rich ecosystems. Therefore, biological communities inhabiting estuaries will show similar features (e.g. species composition, diversity and distribution) to those inhabiting polluted areas, making it difficult to distinguish between natural and anthropogenic stress effects (Dauvin 2007; Elliott & Quintino 2007). This difficulty has been termed as the "Estuarine Quality Paradox" and has been the focus of recent studies dealing with the assessment of ecological status of estuaries (e.g. Borja et al. 2012b; Ellis & Bell 2013; Rakocinski 2012; Veríssimo et al. 2013).

The ecology and taxonomy of benthic diatoms in estuaries

Diatoms (Bacillaryophyta) are the most diverse group of eukaryotic algae, with an estimated number of species on the order of 10^4-10^5 (Mann & Droop 1996). They are distributed worldwide along terrestrial, freshwater and marine habitats, and they often constitute major components of benthic and planktonic algal communities (Smol & Stoermer 2010). Diatoms are characterised by the presence of a siliceous cell wall, formed by two valves that constitute the frustule. The size and shape of the frustule are diagnostic characters used in taxonomy for their identification (Smol & Stoermer 2010).

Benthic diatoms are particularly important in estuarine systems since they are a major component of biofilms (e.g. Méléder et al. 2007), account for up to 50% of total estuarine primary production (Underwood & Kromkamp 1999), have an essential function in food webs (Lamberti 1996), are involved in several biogeochemical cycles e.g. nitrogen and silica cycling (Thornton et al. 2002) and help to stabilise sediments (Underwood & Paterson 1993). Despite a significant body of research on the ecology of estuarine benthic diatoms and their role in the biofilm functioning (reviewed by Trobajo & Sullivan 2010), the elucidation of environmental factors that affect species composition and distribution in the field is not straightforward, mainly because:

i. Physicochemical co-variation inherent in estuaries complicates the identification of environmental parameters directly affecting benthic diatom communities (Underwood et al. 1998). Ecological preferences of freshwater diatoms can be inferred from their distributional patterns in the field (e.g. van Dam et al. 1994), but experimental studies on estuarine diatom species are required to aid in the interpretation of field-based correlations and few such experimental investigations have been made (with a few notable exceptions, e.g. Admiraal 1976, 1977, 1984; Admiraal & Peletier 1979, 1980; Admiraal et al. 1982; Smith & Underwood 2000; Trobajo et al. 2004; Underwood et al.1998; Underwood & Provot 2000, this thesis).

ii. Most abundant diatom species in estuaries have a very difficult taxonomy; partly because they have small cells with a delicate structure (5 - 30 μm) and because they have few (if any)

morphologically distinct taxonomic characters. Moreover, the morphological and morphometric changes occurring during the diatom life cycle (Fig. 3) (e.g. Amato et al. 2005; Armbrust & Chisholm 1992; Trobajo et al. 2006) or as a response to environmental conditions (e.g. Håkansson & Chepurnov 1999; Trobajo et al. 2004; Wood et al. 1987) add more difficulty to their taxonomy. Consequently, the documentation of the diatom species inhabiting estuaries is often poor and confused.

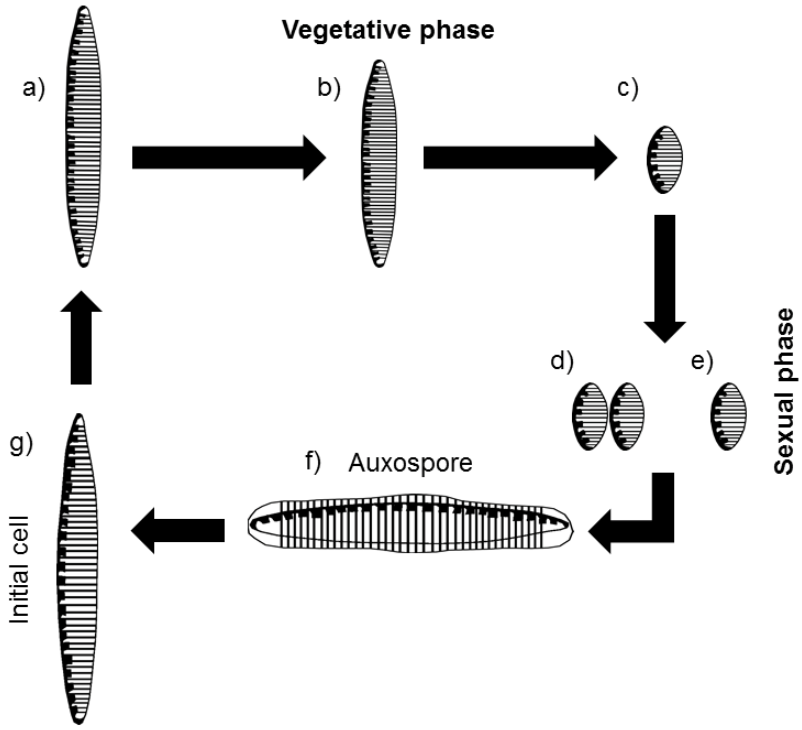

Figure 3. Schematic representation of a life-cycle of a pennate diatom species, with a vegetative phase (a-c), a sexual phase with auxosporulation (d: allogamy, e: automixis), development of an auxospore (f) and with the formation of initial cells (g).

iii. Since there is no comprehensive flora for identifying estuarine diatoms, determinations of species inhabiting these ecosystems requires the use of a sparse and scattered literature, which can be of difficult access. Therefore, estuarine diatoms have been, and are still often identified, by using floras designed for use in freshwaters and applying the criterion of 'the most similar morphology'. Consequently, many estuarine diatoms are identified as species originally described from freshwater ecosystems, without strong evidence that they are truly the same. However, the ecology of most species described from freshwater ecosystems in brackish and/ or marine environments has not been studied.

Any ecological study presupposes the prior taxonomical knowledge of the species inhabiting the target ecosystem. Although the purpose of the present thesis was not taxonomical, the difficulties described above showed that special care and attention had to be given to the taxonomy of estuarine diatoms. Modern taxonomical approaches using molecular techniques and mating experiments have revealed that, though essential, traditional taxonomy based on the morphology of the frustule can be

insufficient and diatom diversity has probably been underestimated (e.g. Mann 2010). Some diatom complexes with very similar or identical morphologies contain two or more genetically and/or reproductively distinct groups (cryptic or pseudocryptic species: e.g. Amato et al. 2007; Beszteri et al. 2005; Créach et al. 2006; Evans et at. 2007; Kooistra et al. 2010; Poulíčková et al. 2010; Trobajo et al. 2009; Vanormelingen et al. 2008). More importantly from an ecological point of view, these cryptic and pseudocryptic species can also show different ecological, geographical and habitat preferences (e.g. Casteleyn et al. 2008; Degerlund et al. 2012; Kaeriyama et al. 2011; Kooistra et al. 2008; Poulíčková et al. 2008; Orsini et al. 2004; Souffreau et al. 2012; Vanelslander et al. 2009). The discovery of a cryptic diversity supposes a new challenge for estuarine diatom ecology, since the wide tolerance to environmental fluctuations shown by most species could be partly caused by the grouping of several species with very similar morphologies but with discrete ecological preferences.

The use of benthic diatoms as bioindicators in estuaries

Macrophytes and phytobenthos are listed as aquatic flora biological quality elements for freshwater ecosystems in the WFD (Annex V), and diatoms have been widely and effectively used as proxies of phytobenthos (Kelly et al. 2008). Their ubiquity, their direct, rapid and sensitive response to physicochemical changes, and their preservation in sediments for a long time makes them good water quality indicators for both present and past environmental changes (Smol & Stoermer 2010). As a result, more than 20 diatom-based indices are routinely used by EU member states to evaluate the ecological status of rivers and streams, with some of them being recently adapted to lakes (Stenger-Kovács et al. 2007).

Although in transitional waters the use of phytobenthos is not obligatory in the WFD procedure (Section 1.2.3 of Annex V), the ecological significance of this community has led to a large number of distributional and functional studies (reviewed in Trobajo & Sullivan 2010). Together with other benthic communities (e.g. macroinvertebrates), benthic diatoms can integrate the effects of several environmental stresses better than other bioindicators such as fish or phytoplankton (Andrén & Jarlman 2008; Hering et al. 2006; Stevenson et al. 2010), which is especially useful in highly dynamic and short-term changing ecosystems such as estuaries. However, while in the case of macroinvertebrates few indices have been adjusted and/or developed for estuarine ecosystems (reviewed in Borja & Dauer 2008, Díaz et al. 2004), there are no specific estuarine diatom-based indices and studies dealing with the application of existing diatom indices in those ecosystems are very scarce (Bauer et al. 2007; Bogaczewicz-Adamczak & Dziengo 2003; Della Bella et al. 2007; Zgrundo & Bogaczewicz-Adamczak 2004).

Study context: The Ebro Estuary

The Ebro basin has a high socioeconomic importance since the Ebro River is the largest river in Spain in terms of water discharge (11.982 hm^3/year before entering the estuary, according to www.chebro.es). Indeed, it is one of the most important rivers of the Mediterranean and has one of the largest catchments in Europe (Tockner et al. 2009). Moreover, the Ebro Delta is one of the largest (330 km^2) deltas in the north-western Mediterranean (Curcó et al. 2002). Human activities in the Ebro River and its estuary have been very intensive during the last two centuries; there has been an intense use of water for industries and cities but mostly for agricultural activities, which caused overexploitation of water resources and high levels of eutrophication until the mid-1990s. Since then, the improvement of urban sewage treatment in the largest cities of the Ebro basin and the reduction of phosphate in detergents has produced a strong decrease in phosphorus concentration with consequent phytoplankton limitation and increase of water transparency (Ibáñez et al. 2008, 2012). This oligotrophication process has produced a shift from a phytoplankton to macrophyte dominance in the river that has triggered other changes in the biological communities. In the Ebro estuary, the main consequence of the decreasing inputs of phosphorus has been a dramatic decrease of phytoplankton in the upper layer (freshwater) and a strong increase in the salt wedge (Falco et al. 2006), which reversed the hypoxic conditions that prevailed in the salt wedge during the period of high eutrophication.

The Ebro River basin has been strongly regulated by a series of reservoirs (approximately 200) mostly constructed between 1940 and 1970. In the lower Ebro River (100 km from the mouth), there are two main reservoirs (Mequinença and Riba-roja, Fig. 5) built in the 1960s for hydropower purposes and with a capacity of 1534 hm^3 and 207 hm^3 respectively. The features and functioning of these reservoirs have affected the hydrology, geomorphology and ecology of the lower Ebro River, with an alteration of timing and magnitude of flows (Batalla et al. 2004), sediment dynamics (Vericat & Batalla 2006; Batalla & Vericat 2009), water temperature and physicochemistry (Ibáñez et al. 1995; Prats et al. 2010; Sabater et al. 2008). The mean annual flow was 592 $m^3\ s^{-1}$ at the beginning of the 20th century, but there has been a decrease since the 1970s (about 400 $m^3\ s^{-1}$ at present) due to irrigation (accounting for 90% of water consumed in the basin) and evaporation in the reservoirs (Fig. 6) (Ibáñez et al. 1996; Ibáñez & Prat 2003). Therefore, the alteration of the hydrological regimes due to the reservoirs and water abstraction is nowadays likely to be the main anthropogenic pressure in the lower Ebro River, with a higher persistence and further upstream extent of the salt wedge as the main consequence in the Ebro estuary.

The present thesis is focused in the last 40 km of the Ebro River, i.e. The Ebro Estuary (40º 43'16.59''N, 0º40'37.79''E). It is considered a "microtidal salt-wedge estuary" and comprises the lower part of the Ebro River, occupying an area within the Ebro Delta of approximately 10 km^2.

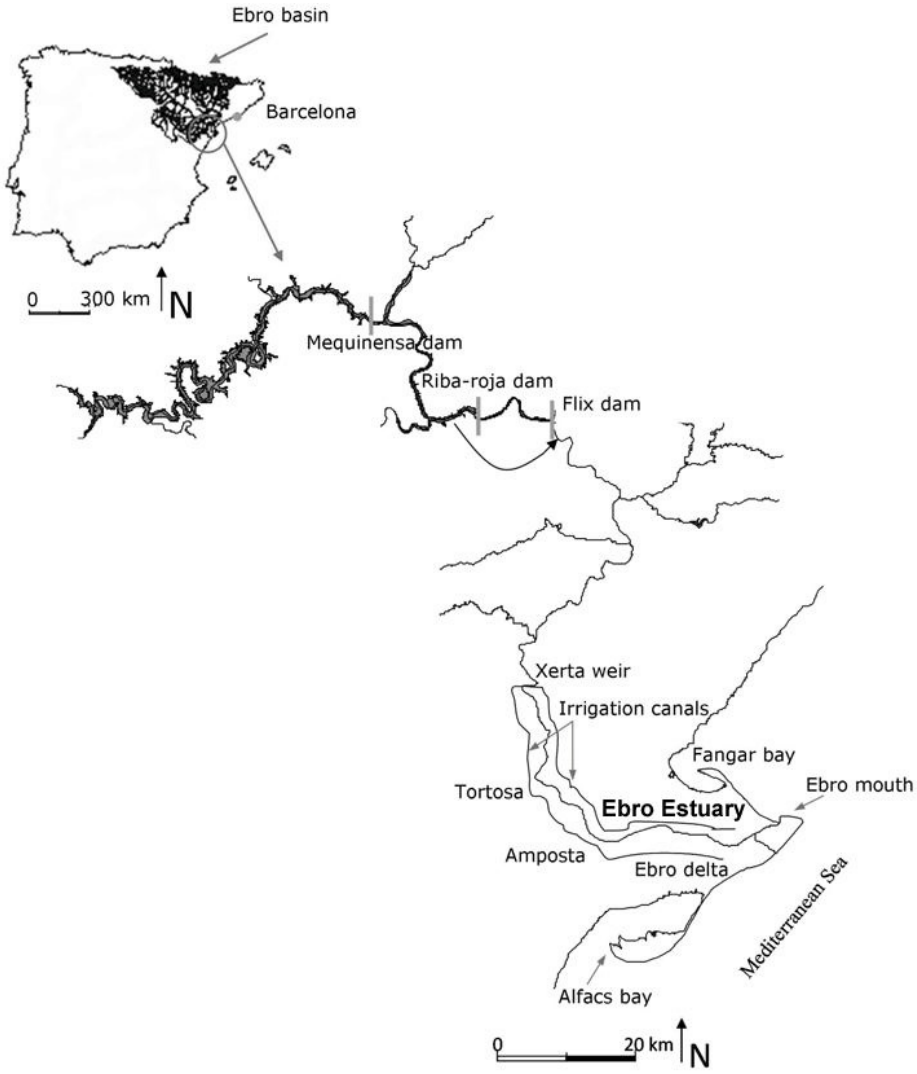

Figure 5. The lower Ebro River, the Ebro Estuary and the Ebro Delta, with the main localities and reservoirs (Mequinença, Riba-Roja and Flix). Extracted from http://www.deltanet-project.eu/workshop-1-report-flood-risk-and-sediment-management.

The Ebro Estuary and its Delta are under coastal Mediterranean-type climate, characterised by moderate winters (average monthly temperature of 9.18 ºC in January) and sub-arid summers (average monthly temperature of 25.68 ºC in August). Rainfall (average annual of 548 mm) is very scarce in summer and concentrated in fall, although interannual variability is very high. Strong winds during February-May and September-December can increase the range of water level fluctuations from 0.2 m (the usual) to > 1 m (Curcó et al. 2002).

The Ebro Estuary is the most studied Mediterranean salt-wedge estuary, though most of the research has been focused on the hydrological functioning of the salt wedge and nutrient and phytoplankton dynamics and composition (Casamayor et al. 2001; Falco et al. 2006, 2010; Guillén & Palanques 1992; Ibáñez et al. 1995, 1997, 1999; Pérez et al. 2002, 2009; Sierra et al. 2002, 2004). The increase in water transparency and the shift from a eutrophic to a mesotrophic ecosystem has improved the light and nutrient conditions in the salt wedge, which enhanced the establishment of

benthic communities. However, despite the ecological and socioeconomic importance of the Ebro estuary, studies of its benthic biota are still very scarce, from which only macroinvertebrate community has been described (Ibáñez et al. 1995; Nebra et al. 2011).

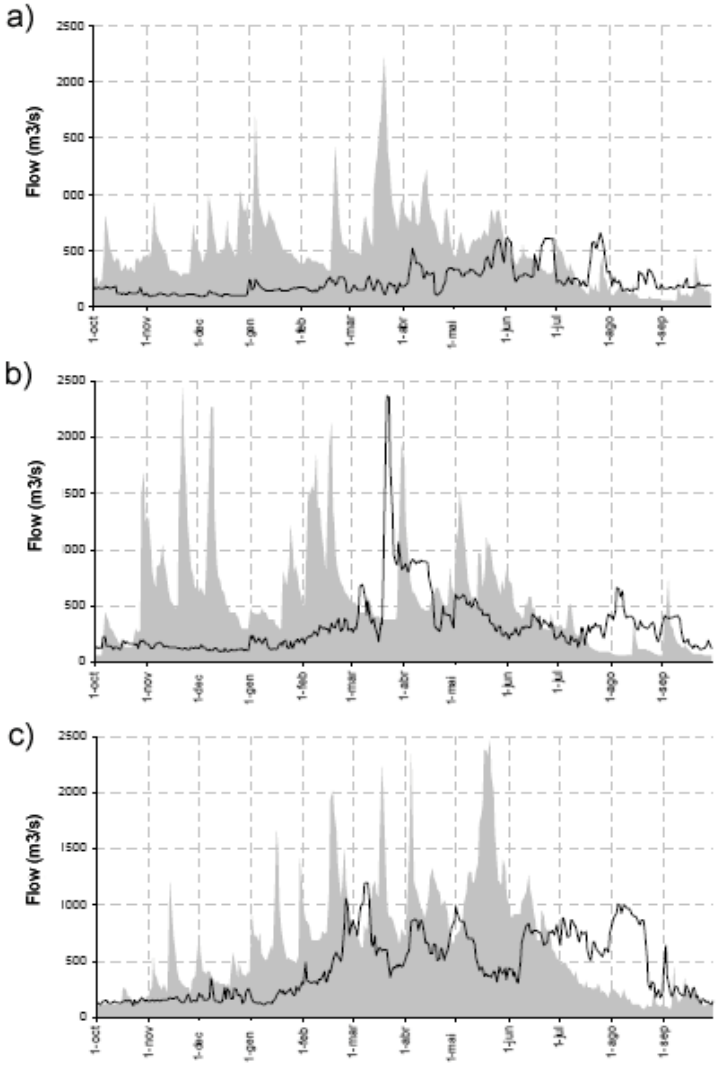

Figure 6. Monthly Ebro River flow at Tortosa (40 km from mouth) during historical conditions before reservoirs construction (grey) and in the present (black line). Comparison between dry (a), medium (b) and wet (c) years. Extracted from Sánchez & Ibáñez (2008).

This thesis is the first study on the benthic diatom community of the Ebro Estuary and arises as a consequence of the need to evaluate its ecological status under the WFD. It is part of a broader study on the ecological features of benthic communities (i.e. macroinvertebrates and benthic diatoms) and their use as bioindicators in the Ebro Estuary, which has been funded by the Catalan WaterAgency (ACA). A multidisciplinary approach has been adopted, where field-based data has been combined with taxonomical, experimental and phylogenetic studies.

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Objectives

The general aim of the thesis was to improve knowledge of the ecology and taxonomy of estuarine benthic diatoms in a highly stratified Mediterranean estuary, in order to evaluate their potential use as bioindicators of this ecosystem. To achieve that, diatom community composition was described and the factors affecting its composition and distribution were elucidated (**Chapter 1**). Once the main anthropogenic pressures in the Ebro Estuary had been established, the response of diatoms to these pressures was tested through: i) the evaluation of existing diatom indices, and ii) the identification of groups of species indicative of potentially altered environmental conditions (**Chapter 2**). Field studies were supported with experimental studies to test the response of species to the main environmental gradient in the Ebro Estuary (i.e. salinity) (**Chapter 3 and 5**). Several taxonomical aspects of estuarine diatoms were studied in detail to aid in the understanding and interpretation of ecological results. First of all, the morphological variability and ecophysiological response of selected species to salinity was documented (**Chapter 3**). The morphology of the valves of several small and morphologically similar diatom species from the genus *Nitzschia* (one of the most abundant in the Ebro Estuary) was compared and studied in detail (**Chapter 4**). Finally, the thesis focused on a species complex, i.e. *Nitzschia inconspicua,* to analyse the morphological, genetic, reproductive and ecophysiological variability of a taxonomically difficult but ecologically important species of both freshwater and transitional waters (**Chapter 5**). Several specific questions were addressed in each of the thesis chapters:

Chapter 1:

What is the composition and structure of the benthic diatom community of the Ebro Estuary? Which environmental gradients determine benthic diatom composition and distribution in the Ebro Estuary? Is there any spatial or temporal pattern in diatom community assemblages? Is benthic diatom community affected by other abiotic factors (e.g. natural versus artificial substrata)?

Chapter 2:

Which are the main human pressures in the Ebro Estuary? Are benthic diatoms potentially good bioindicators of altered conditions in the Ebro Estuary? Is there any group of diatom species that could be used as indicators of human pressures? Is it feasible and reliable to apply existing diatom-based indices to evaluate the ecological status of the Ebro Estuary and/or other transitional waters?

Chapter 3:

Which are the effects of salinity on the growth and valve morphology of some estuarine diatom species? Do these effects have a repercussion at taxonomical level? Do the studied

diatom species have the same degree of tolerance to the salinity treatments? Is there any pattern of morphological and/or growth variation shared by the different species in response to salinity?

Chapter 4:

Is it possible to distinguish some of the small and taxonomically complex *Nitzschia* species, frequent and abundant not only in the Ebro Estuary but also in many other ecosystems? Is it feasible to correctly identify these *Nitzschia* species in future field-based works? Do these species have distinct ecophysiologies that justify the need to properly identify them in future biomonitoring methodologies?

Chapter 5:

Which are the factors that make *N. inconspicua* such a taxonomically difficult diatom with confusing ecology, despite its abundance in many aquatic ecosystems and correspondingly abundant opportunities for study? Is there any morphological pattern between diferent populations belonging to *N. inconspicua*? Is there any relationship between morphological, ecophysiological, reproductive and molecular variability? Is *N. inconspicua* a single species with a wide ecological spectrum or is it composed by several cryptic species, each with narrower ecological preferences with regard to salinity?

List of publications

The following five publications constituted the main body of this thesis, in which the ecology and taxonomy of estuarine benthic diatoms in the Ebro Estuary and their use as bioindicators is investigated through a multidisciplinary approach:

Article 1:

Rovira, L., Trobajo, R., Leira, M. & Ibáñez, C. (2012) The effects of hydrological dynamics on benthic diatom community structure in a highly stratified estuary: The case of the Ebro Estuary (Catalonia, Spain). **Estuarine, Coastal and Shelf Science** 101, 1-14.

Article 2:

Rovira, L., Trobajo, R. & Ibáñez, C. (2012) The use of diatom assemblages as ecological indicators in highly stratified estuaries and evaluation of existing diatom indices. **Marine Pollution Bulletin** 64, 500-511.

Article 3:

Trobajo, R., **Rovira, L.**, Mann, D.G. & Cox, E.J. (2011) Effects of salinity on growth and on valve morphology of five estuarine diatoms. **Phycological Research** 59, 83-90.

Article 4:

Trobajo, R., **Rovira, L.**, Ector, L., Morales, E., Wetzel, C. E., Kelly, M. & Mann, D.G. (2012) Morphology and identity of some ecologically important small *Nitzschia* species. **Diatom Research** 28 (1), 37-59.

Article 5:

Rovira, L., Trobajo, R., Sato, S., Ibáñez, C. & Mann, D.G. Genetic and ecophysiological diversity in the diatom *Nitzschia inconspicua*. **Journal of Phycology** (under review).

However, during the research period of this thesis, several parallel questions arised with the publication of additional articles that are enclosed here in the Appendix:

Appendix:

Rovira, L., Trobajo, R. & Ibáñez, C. (2009) Periphytic diatom community in a Mediterranean salt-wedge estuary: the Ebro Estuary (NE Iberian Peninsula). **Acta Botanica Croatica** 68 (2), 285-300.

Rovira, L., Witkowski, A.,Trobajo, R., Ibáñez, C. & Ruppel, M. (2011) *Planothidium iberense* spec. nov., a new brackish diatom of the Ebro Estuary (NE Spain). **Diatom Research** 26 (1), 99-107**.**

Mann, D.G., Sato, S., **Rovira, L.**, & Trobajo, R. (2013) Paedogamy and auxosporulation in *Nitzschia* sect. *Lanceolatae* (Bacillariophyta). **Phycologia** 52 (2), 204-220.

Chapter 1

Rovira, L., Trobajo, R., Leira, M. & Ibáñez, C. (2012) **The effects of hydrological dynamics on benthic diatom community structure in a highly stratified estuary: The case of the Ebro Estuary (Catalonia, Spain).** Estuarine, Coastal and Shelf Science 101, 1-14.

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The effects of hydrological dynamics on benthic diatom community structure in a highly stratified estuary: The case of the Ebro Estuary (Catalonia, Spain)

L. Rovira [a,*], R. Trobajo [a], M. Leira [b], C. Ibáñez [a]

[a] IRTA - Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540, St. Carles de la Ràpita, Catalonia, Spain
[b] University of Vigo - Faculty of Sciences, Department of Plant and Soil Science, Campus As Lagoas, 32004, Ourense, Galicia, Spain

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ABSTRACT

This study of the distribution of benthic diatom assemblages and their relationship with environmental factors in a highly stratified Mediterranean estuary, i.e. the Ebro Estuary, shows the importance of hydrological dynamics to explain the features of the diatom community in such an estuary, where river flow magnitude and fluctuations imply strong physicochemical variability especially in sites close to the sea. Eight sites along the estuary were sampled during 2007–2008 both at superficial and deep water layers, in order to gather both horizontal and vertical estuarine physicochemical and hydrological gradients. Canonical Variates Analysis and Hierarchical Cluster Analysis segregated diatom community in two assemblages depending on the dynamics of the salt-wedge. The diatom assemblages of riverine conditions (i.e. without salt-wedge influence) where characterised by high abundances of *Cocconeis placentula* var. *euglypta* and *Amphora pediculus*, meanwhile high abundances of *Nizschia frustulum* and *Nitzschia inconspicua* were characteristic of estuarine conditions (i.e. under salt-wedge influence). Redundancy Analysis showed that both diatom assemblages responded seasonally to Ebro River flows, especially in estuarine conditions, where fluctuating conditions affected diatom assemblages both at spatial and temporal scale.

1. Introduction

Estuaries and coastal wetlands are highly dynamic systems characterised by pronounced natural gradients in environmental properties due to transition between continental and marine systems. This dynamism implies a strong fluctuation of the main environmental parameters (e.g. salinity, nutrients, oxygen, water turnover), which affects the distribution and composition of the biological communities. Because of these intrinsic characteristics, estuaries are complex systems to study, although this natural variability makes them very valuable in order to elucidate the forces driving biological communities (Trobajo et al., 2004a). Salt-wedge estuaries are characterised by a strong vertical stratification in situations of low river flows, whereas this salt-wedge disappears when river flow is high (Ibáñez et al., 1997). This type of estuary is common in low tidal amplitude seas (e.g. the Mediterranean), such as the Ebro Estuary (Ibáñez et al., 1999; Sierra et al., 2002) and those from the eastern Adriatic coast (Burić et al., 2004; Cetinić et al., 2006), some examples of microtidal highly stratified estuaries.

Benthic diatoms have an important function in estuarine systems since they are major component of the biofilms (Méléder et al., 2007), contribute highly to primary production (up to 50%) (Underwood and Kromkamp, 1999), have an essential function in food webs (Lamberti, 1996), are involved in different biogeochemical cycles (e.g. nitrogen and silica cycling) (Thornton et al., 2002) and help to stabilise sediments (Underwood and Paterson, 1993). Diatoms have also been considered as excellent water quality indicators for both past and present conditions of water bodies because they respond directly and sensitively to many physical, chemical and biological changes and their silica wall can be preserved in sediments for a long time. As an advantage over other bioindicators, they have shorter generation times, showing a rapid response to environmental changes and therefore become early warning indicators of changes in nutrient status (Smol and Stoermer, 2010).

The diatom community in estuaries is subjected to natural fluctuations in hydrological and chemical parameters resulting in variability on species composition and biomass both at spatial and temporal scale (Admiraal and Peletier, 1980; Underwood and Kromkamp, 1999; Guarini et al., 2008; Van der Wal et al., 2010). Due to this intrinsic complexity of estuarine systems, studies on diatom communities of estuaries are scarce compared to those of

* Corresponding author.
E-mail address: laia.rovira.torres@gmail.com (L. Rovira).

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freshwater systems. Most studies on the ecology of benthic diatom communities in estuaries have been focused on tidal estuaries with well-mixed water layers (McIntire and Overton, 1971; Baillie, 1987; Underwood, 1994; Peletier, 1996; Muyalert et al., 2002; Chindah, 2004; Frankovich et al., 2006; Gordon et al., 2008); the work of Gómez et al. (2009) in La Plata River Estuary (Argentina) is the only one dealing with benthic diatoms in a microtidal estuary, although this focuses only on the freshwater tidal zone.

The Ebro Estuary is a Mediterranean microtidal estuary (tide range around 20 cm), highly stratified into two water layers, with the presence of a salt-wedge during most of the year (Ibáñez et al., 1997). Research on the Ebro Estuary during the last 20 years has been focused on hydrological studies of salt-wedge dynamics (Ibáñez et al., 1997, 1999; Sierra et al., 2002, 2004), and of nutrients and phytoplankton (Ibáñez et al., 1995; Casamayor et al., 2001; Pérez et al., 2002, 2009; Falco et al., 2006, 2010). Despite the importance of the Ebro River (it is the largest river in Spain and its basin supports an intensive water use) (Ibáñez and Prat, 2003), there is a lack of studies on its estuarine benthic communities, with the exception of very few studies on its macroinvertebrate community (Ibáñez et al., 1995; Nebra et al., 2011) and a preliminary study on benthic diatoms (Rovira et al., 2009).

The aim of this study was to explain the structure and dynamics of the benthic diatom community of the Ebro Estuary as a function of the main environmental factors both at spatial and temporal scales, and to identify the main benthic diatom assemblages that characterise the prevailing ecological conditions in the estuary.

2. Materials and methods

2.1. Study area

The Ebro Estuary is 40 km long with a mean width of 237 m and a mean depth of 6.8 m. It covers an approximate area of 10 km^2 and it is considered a "micro-tidal salt-wedge estuary". The maximum intrusion of the salt-wedge into the Ebro River is of 32 km. The river flow, which is the main factor affecting the hydrological dynamics of the estuary, has been highly regulated by dams built since the 1960s, in particular two reservoirs built at 100 km upstream of the mouth for hydropower purposes (Mequinensa and Riba-roja reservoirs). This river flow regulation increased the presence of the salt-wedge in the estuary, being persistent during most of the year. The salt-wedge disappears when the Ebro River flow is above 400 $m^3\ s^{-1}$. Between 250 and 400 $m^3\ s^{-1}$ the salt-wedge occupies the last 5 km of the estuary, and with flows below 250 $m^3\ s^{-1}$, the salt-wedge advances up to 18 km from the river mouth. When the river flow is less than 100 $m^3\ s^{-1}$, the salt-wedge reaches its maximum extent, i.e. 32 km from the river mouth (Ibáñez et al., 1997), though this situation is much less frequent than the above mentioned ones.

2.2. Sampling

Sampling campaigns were conducted every three months from October 2007 to December 2008 in 8 sites distributed every 3–6 km along the estuary (Fig. 1). Sampling sites were chosen to gather estuarine gradients, from upstream sites above the maximum extent of the salt-wedge to downstream sites located in the river mouth. At all sampling sites, water depth, temperature, electrical conductivity (EC_{25}), dissolved oxygen (DO_2) and pH were measured in situ with a YSI 556 multiprobe. Flow direction and velocity were also measured in situ using a Braystoke BFM 001 current flow meter. Irradiance was measured with a QSP-2100 Submersible Scalar PAR Sensor. River flow records (measured at Tortosa, 40 km upstream from the river mouth) were obtained from the Ebro Water Authority (CHE) database. The data from 30 days before sampling were used to calculate maximum, minimum, mean and fluctuation (difference between maximum and minimum) of Ebro River flow.

Benthic diatom samples were collected every three months from both natural substrata (mainly the macrophytes *Potamogeton pectinatus* and *Ceratophyllum* spp., and wood debris where macrophytes were not available) and artificial substrata (fired clay bricks). Fired clay bricks were placed at superficial (0.5 m) and deep

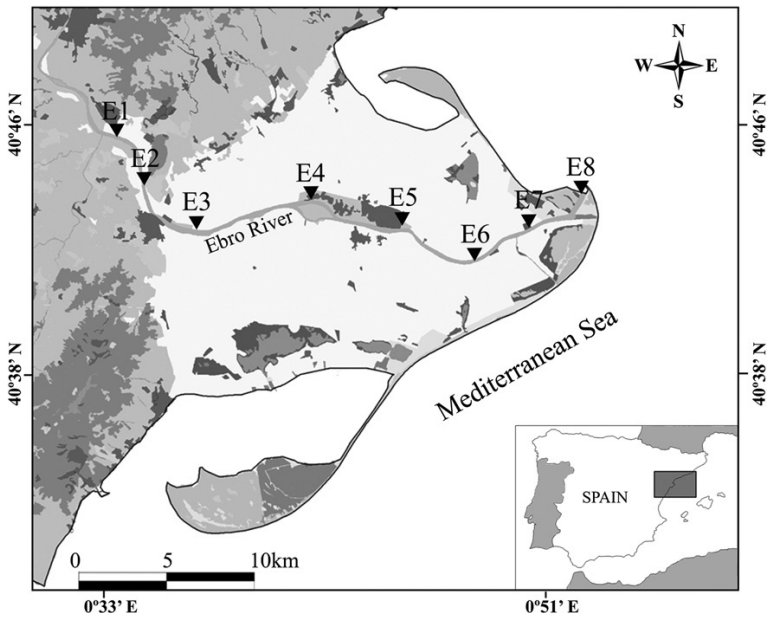

Fig. 1. Ebro Estuary map showing the sampling sites.

(4–6 m) levels at each site, they were collected after three months of colonisation and replaced with new ones every sampling campaign. This sampling design allowed the gathering of both vertical and horizontal physicochemical gradients in the estuary. Artificial substrata were considered sufficiently robust to resist high flows and sudden flow fluctuations that characterise the lower Ebro River. However, due to the occurrence of floods, some samples of artificial substrata were not recovered during the sampling period. An area of 4 cm^2 was scraped off the artificial substrata and three fragments from natural substrata were included in each replicate. Samples were fixed in 4% formaldehyde solution and two replicates from both artificial and natural substrata were processed.

2.3. Diatom identification

Benthic samples were oxidised with H_2O_2 30% v/v for several hours in order to remove the organic matter. HCl^- 37% v/v was added to evaporate the carbonates from the samples, as described in Renberg (1990). Clean valves were permanently mounted with Naphrax© (refractive index 1.74). The permanent slides were examined using a LEICA DMI 3000 B light microscope equipped with differential interference contrast (DIC) with a 100 times oil immersion objective (n.a = 1.40). For scanning electron microscopy (SEM) examinations, cleaned material was filtered onto Nuclepore Whatman polycarbonate membranes. Filters were air-dried overnight, mounted onto aluminium stubs, coated with gold-palladium and examined in a Hitachi S-5500 SEM apparatus.

A minimum of 400 valves were counted in each substratum replicate. Diatom abundance is presented as relative percentages. Identification of diatoms to species level followed Krammer and Lange-Bertalot (1986, 1988, 1991a, b); Witkowski et al. (2000) and Lange-Bertalot (2001), though many other taxonomic and floristic works were also used.

2.4. Nutrients and chlorophyll analysis

Superficial and deep water samples were collected at each sampling site. Inorganic dissolved nutrients: silicate ($Si{-}SiO_4^{4-}$), nitrate ($N{-}NO_3^-$), nitrite ($N{-}NO_2^-$), phosphate ($P{-}PO_4^{3-}$); total nitrogen (TN) and total phosphorus (TP) were measured following Grasshoff et al. (1999), while ammonium ($N{-}NH_4^+$) was measured following the method proposed by the equipment manufacturer, ALLIANCE INSTRUMENTS, SA. Total suspended solid concentration (TSS) and particulate organic matter (POM) were quantified according to the UNE-EN 872 norm (AENOR, 1996). Phytoplankton chlorophyll *a* was extracted using 90% acetone after filtering water and measured with a fluorimeter using the Lorenzen formula (Lorenzen, 1966).

2.5. Data analysis

A Hierarchical Cluster Analysis was performed in order to group the samples according to their similarity in diatom species composition. Sorensen's similarity coefficient was measured on relative abundance data, and flexible beta (−0.25 according to Dufrêne and Legendre, 1997) was selected as the linkage method. The statistical significance of between group's differences was tested using the Multi-response Permutation Procedure (MRPP). MRPP is a non-parametric procedure (i.e. it does not require normality and homogeneity of variances) that tests the hypothesis of no differences in assemblage structure among groups. Sorensen's coefficient was also used as the distance measure. Cluster analysis and MRPP procedure were run with PcOrd 5.0 for Windows.

Similarity Percentage Analysis (SIMPER) was performed to identify diatom species responsible for the similarity within clusters. SIMPER analysis compares the average contribution of each diatom species to the average Bray–Curtis similarity within samples of a cluster. The species which consistently contribute greatly to the average similarity between samples (those that occur at a constant high abundance and frequency) are considered characteristic of the cluster. SIMPER was run with PRIMER 6.0 software for Windows.

Multivariate statistical techniques were also used to explore the relationship between the diatom community distribution and the main environmental parameters. Diatom data were analysed with Detrended Correspondence Analysis (DCA) in order to determine the length of the gradient. DCA analysis indicated that the gradient length was greater than 2 sd units (2.592). Hence, the use of unimodal ordination techniques would be appropriate for our data (Lepš and Šmilauer, 2003). DCA (an indirect gradient ordination based on the unimodal response of the species to the environment) was therefore used to determine the major patterns of variation of the samples based on differences in species composition, without incorporating environmental data.

Canonical Variates Analysis (CVA) was used to relate established clusters to all measured environmental parameters. Furthermore, diatom assemblages of different environmental conditions (clusters 1 and 2; clusters 3 and 4) were analysed separately in order to elucidate their response to the same environmental data. Since gradient length of distinct diatom assemblages never exceeded 2 sd units when they were analysed separately, linear ordination techniques such as Redundancy Analysis (RDA) were applied. In both CVA and RDA analyses, environmental data (except for pH) was logarithmically transformed before analysis to reduce skewed distributions. According to the preliminary analyses, collinear variables (with a VIF gt; 20) were found and therefore excluded from the final analyses. Step-wise forward selection and a Monte Carlo permutation test were used to choose only significant variables ($P < 0.05$). Probabilities for multiple comparisons were corrected using the Bonferroni correction. In both RDA sets, the remaining environmental variables were added as supplementary variables. All ordinations analyses were performed using CANOCO v. 4.5 for Windows.

Finally, the environmental parameters that accounted for a significant proportion of CVA were correlated with the remaining physicochemical parameters by Spearman's correlations ($P < 0.01$) using SPSS software. Only species with a relative abundance (RA) > 0.2% and present in more than 5% of all the samples were included in all the analyses.

3. Results

3.1. Environmental characteristics

Table 1 show the mean values and variation ranges for the water physicochemical parameters at each sampling site. Maximum, minimum, mean and fluctuation of the Ebro River flow are shown in Table 2. The limit of salt-wedge intrusion was situated between sampling sites E4 and E5 (depending on the Ebro River flow) during most of the campaigns, except for December 2008 (river flow was above 500 $m^3\ s^{-1}$), when the salt-wedge was not present. Because of the stratification of water layers (due to the microtidal range regime) and the advective circulation, the Ebro Estuary shows longitudinal gradients both at superficial and deep water layers due to the proximity to the sea. Thus, conductivity increased downstream in both layers, being oligohaline (EC_{25} grading from 0.75 mS cm^{-1} to 6.99 mS cm^{-1}) in the superficial layer and ranging from oligohaline to euhaline (EC_{25} from 0.75 mS cm^{-1} to 54.17 mS cm^{-1}) in the deep layer (Table 1).

The stratification of water layers gave a vertical gradient in the estuary where the salt-wedge was present. The deep water layer of

Table 1
Annual mean, minimum, maximum and coefficient of variation (CV) of water physico chemical parameters measured in the Ebro Estuary. The negatives values in water velocity mean that water flowed in opposite direction to river flow. S = superficial water layer, D = deep water layer. Sampling sites where the salt-wedge was present most part of the year are highlighted in light grey. The mean of pH was calculated as the mean of $[H^+]$ and converted back to a pH value afterwards.

Site	Temp (°C)				pH				DO_2 (mg/L)				Conductivity (mS/cm)				$P{-}PO_4^{3-}$ (μg/L)				TP (μg/L)				$N{-}NH_4^+$ (μg/L)				$N{-}NO_2^-$ (μg/L)			
	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV
E1S	18.3	11.0	24.6	33	8.04	7.84	8.19	2	8.9	7.1	13.7	29	1.03	0.76	1.38	23	34.1	9.6	49.9	23	59.4	36.5	74.5	44	60.8	24.2	87.1	22	17.5	4.2	34.8	39
E1D	18.3	11.0	24.5	33	8.06	7.88	8.20	1	8.7	6.9	13.2	27	1.03	0.76	1.37	23	34.4	10.9	47.2	23	68.6	39.9	110.6	38	58.8	24.5	145.2	35	17.0	4.5	32.9	75
E2S	18.5	11.7	24.8	33	8.09	7.89	8.25	2	8.9	7.7	13.7	26	1.03	0.75	1.37	23	33.6	12.3	49.5	22	63.1	36.0	103.0	39	71.8	58.5	104.3	37	17.7	5.2	34.5	23
E2D	18.4	11.4	24.6	33	8.09	7.94	8.24	1	8.7	7.0	13.7	29	1.03	0.75	1.37	23	30.2	11.6	42.6	22	58.4	42.2	84.7	38	71.9	44.5	98.5	28	18.1	7.6	35.8	25
E3S	18.5	11.6	24.8	33	8.11	7.96	8.34	2	9.8	7.3	16.5	35	1.05	0.76	1.37	22	30.1	12.1	48.6	22	52.3	35.6	74.0	72	72.4	22.4	126.9	53	17.0	5.2	34.2	83
E3D	18.4	11.4	24.5	33	8.11	7.98	8.32	2	9.3	7.2	14.8	30	1.05	0.76	1.37	23	29.9	9.7	52.9	23	50.6	36.4	60.6	59	110.2	18.5	391.4	22	21.1	5.4	52.9	126
E4S	18.3	10.8	25.1	35	8.11	7.85	8.37	2	10.4	7.6	17.7	36	1.15	0.97	1.41	16	31.2	9.4	57.7	21	48.3	31.1	80.2	55	125.1	7.0	472.5	36	22.5	8.7	55.5	139
E4D	18.4	10.8	23.7	32	7.98	7.85	8.21	2	6.4	2.2	10.3	57	32.46	1.00	52.57	76	36.5	19.4	62.9	75	73.3	33.5	114.6	45	213.1	9.6	720.6	44	21.4	5.5	55.0	126
E5S	18.4	10.8	25.2	34	8.14	7.92	8.35	2	10.2	7.6	19.1	43	1.68	1.00	2.05	22	31.2	7.7	64.9	21	47.6	26.3	75.8	69	114.0	27.2	370.8	36	21.5	11.1	48.8	113
E5D	17.9	10.8	23.4	31	8.03	7.72	8.30	3	6.9	3.7	11.3	45	39.76	1.01	53.37	50	22.6	8.5	43.4	48	57.7	20.0	90.7	70	57.6	32.9	100.0	49	17.6	1.8	55.5	46
E6S	18.3	10.8	25.0	34	8.13	7.98	8.34	2	9.6	7.5	15.6	32	2.49	0.97	3.31	33	30.1	10.1	56.0	29	52.2	36.3	93.9	58	99.2	31.4	316.4	40	19.5	10.6	43.1	110
E6D	17.9	10.8	23.4	31	8.06	7.75	8.33	3	7.2	5.2	10.4	33	40.20	0.97	53.82	50	21.1	3.8	41.5	48	51.2	14.6	74.1	74	35.2	11.0	65.3	44	11.7	0.3	38.8	61
E7S	18.2	10.7	25.0	34	8.16	8.05	8.35	1	10.5	7.3	20.5	47	2.90	1.18	3.78	33	29.6	10.3	50.5	27	48.4	35.9	70.2	58	95.3	40.4	177.8	24	18.4	10.5	33.9	54
E7D	17.9	10.7	23.4	31	8.10	7.86	8.35	2	7.7	5.6	10.6	29	40.46	1.25	53.61	49	15.8	1.7	35.3	47	44.0	14.8	67.4	90	27.3	4.1	53.8	41	9.9	0.3	24.6	61
E8S	18.0	10.6	25.1	34	8.10	7.97	8.30	2	9.6	7.5	13.6	23	4.28	1.26	6.99	44	30.1	9.1	46.0	36	52.4	37.9	77.1	50	123.6	46.8	372.9	25	18.5	10.3	33.9	101
E8D	17.9	10.8	23.7	30	8.12	7.95	8.42	2	8.4	7.0	10.4	14	44.77	29.21	54.17	21	21.4	0.6	47.2	12	47.4	12.1	67.4	96	59.2	22.1	164.2	44	10.8	0.7	37.4	90

Site	POM (%)				$Si{-}SiO_4^{4-}$ (mg/L)				Water velocity (m/s)				Water chl *a* (μg/L)				TSS (mg/L)				$N{-}NO_3^-$ (mg/L)				TN (mg/L)				Distance from the sea (Km)
	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	
E1S	35.9	16.2	68.9	50	1.4	0.9	2.9	56	0.5	0.3	0.8	51	0.73	0.50	0.99	29	3.3	1.5	5.8	49	2.9	1.6	4.5	58	3.8	2.1	6.6	41	33.4
E1D	37.5	15.9	74.4	58	1.3	0.6	2.7	55	0.2	0.1	0.4	43	0.92	0.59	1.17	23	3.5	1.0	6.0	61	2.8	1.6	4.4	55	3.9	2.1	7.0	41	33.4
E2S	35.0	14.9	65.7	55	1.4	0.8	2.7	52	0.2	0.1	0.5	65	0.68	0.45	1.03	31	3.9	2.6	6.2	38	2.9	1.6	4.6	56	3.9	2.1	6.5	42	29.0
E2D	27.9	14.7	44.7	40	1.2	0.6	2.8	64	0.2	0.1	0.4	67	1.30	0.71	2.88	65	5.4	3.1	8.7	38	2.9	1.7	4.6	53	3.8	2.2	6.9	43	29.0
E3S	25.5	0.4	41.5	59	1.9	0.9	3.8	58	0.3	0.2	0.6	59	0.73	0.49	1.07	31	4.5	0.4	6.9	59	2.3	1.6	3.9	129	3.8	2.1	6.6	43	24.0
E3D	22.6	14.6	31.6	28	1.4	1.0	2.3	33	0.4	0.1	1.1	108	0.97	0.57	1.50	43	6.4	2.7	13.1	69	2.8	1.6	4.3	79	3.9	2.0	7.1	40	24.0
E4S	36.0	12.6	59.6	48	1.3	1.0	1.6	16	0.3	0.2	0.7	57	1.21	0.64	2.47	58	4.9	1.8	10.3	64	2.8	1.6	4.3	75	3.8	2.1	6.2	39	18.3
E4D	26.4	19.4	46.3	35	1.0	0.3	1.6	54	0.1	−0.1	0.3	226	1.79	0.50	5.08	104	7.1	2.3	22.1	107	1.3	0.0	4.1	84	2.1	0.1	5.6	142	18.3
E5S	30.5	17.8	48.6	33	1.5	1.0	2.1	30	0.3	0.2	0.6	47	1.56	0.42	4.07	92	5.7	2.0	17.0	100	2.8	1.5	4.4	65	3.7	2.0	6.1	45	12.3
E5D	27.5	14.4	40.7	40	0.6	0.2	1.6	87	0.1	−0.1	0.4	241	3.16	0.52	14.50	176	6.5	2.1	17.1	93	1.2	0.0	3.4	114	1.9	0.1	5.8	145	12.3
E6S	39.1	12.3	89.1	70	1.4	0.8	2.6	44	0.4	0.3	0.6	31	1.16	0.38	2.86	82	3.9	2.1	10.2	82	2.7	1.5	4.2	64	3.6	1.9	6.3	44	6.6
E6D	24.0	13.2	37.1	38	0.7	0.1	2.1	118	0.0	−0.1	0.4	816	1.74	0.45	3.79	70	5.9	2.5	17.1	94	1.2	0.0	3.9	124	1.9	0.1	6.7	147	6.6
E7S	33.6	13.2	60.0	50	1.5	0.9	2.5	39	0.6	0.4	0.8	25	1.19	0.38	3.06	84	3.7	2.7	5.3	25	2.6	1.4	3.9	48	3.6	2.0	6.2	44	3.6
E7D	22.8	12.5	31.6	35	0.5	0.1	0.9	67	0.0	−0.1	0.2	566	1.89	0.43	3.96	74	4.5	2.6	6.5	40	0.9	0.0	3.2	114	1.6	0.1	6.6	150	3.6
E8S	32.8	14.7	66.2	56	1.2	0.7	2.1	47	0.5	0.4	0.6	13	1.06	0.47	1.76	58	3.7	2.8	6.0	32	2.6	1.4	3.8	50	3.7	2.1	6.4	43	0
E8D	17.5	7.8	31.3	53	0.7	0.1	1.2	76	0.0	−0.2	0.1	686	1.42	0.28	3.02	82	25.1	2.9	75.1	117	1.2	0.0	3.9	128	1.7	0.1	5.0	136	0

Table 2
Mean, minimum (Min.), maximum (Max.) and fluctuation of Ebro River flow during 30 days before sampling campaigns.

	Ebro River flow (m^3/s)			
	Mean	Min.	Max.	Fluctuation
October 2007	177.80	123.70	263.47	139.77
January 2008	108.56	93.45	132.80	39.35
April 2008	194.08	101.60	469.16	367.56
July 2008	342.25	92.68	877.47	784.80
September 2008	180.79	137.80	269.00	131.20
December 2008	348.69	136.20	757.62	621.42

this area (sampling sites E4-E8) not only showed the highest conductivity values but also showed lower DO_2, $P{-}PO_4^{3-}$, TN and $Si{-}SiO_4^{4-}$ concentration than the superficial water layer (Table 1, Fig. 2). Sampling site E4 did not follow this pattern for $P{-}PO_4^{3-}$ and $N{-}NH_4^+$ concentrations, being higher at the deep layer than at the superficial layer (Fig. 2e–f).

3.2. Diatom community

A total of 160 diatom species were found in the samples analysed. From these, only 62 species had a relative abundance higher than 0.2% and were present in more than 5% of the samples (Table 3), therefore they were used in the statistical analyses. The most abundant taxa belonged to widespread genera as *Cocconeis*, *Nitzschia*, *Amphora* and *Rhoicosphenia*. *Cocconeis placentula* var. *euglypta* (Ehrenberg) Grunow was the most abundant species and occurred in 99% of the samples. *Navicula* was the genus represented by the highest number of species (34), followed by *Nitzschia* (25).

Cluster analysis produced 4 diatom assemblages according to the similarity in species composition (Fig. 3). The MRPP indicated significant differences between the identified groups ($A = 0.199$, $P = 0.001$). Physicochemical characteristics of each cluster are shown in Table 4; cluster 1 (31 cases) and cluster 2 (27 cases) mainly corresponded to diatom assemblages from upstream sites (E1, E2, E3, most of the E4 superficial samples), with the exception of one E5 and four E7. These sites presented weak superficial marine influence, no salt-wedge intrusion and therefore rather well-mixed water column (except for 1 sample in cluster 2, where salt-wedge was present). Most of the October 2007 samples were grouped together in cluster 1 whereas April 2008 samples were gathered in cluster 2. Cluster 3 (17 cases) and cluster 4 (21 cases) incorporated samples from downstream sites (most E4D, some E5S, E5D, E6, most E7 and E8), with marine influence both at superficial and deep water layers, resulting in a stratification of the water

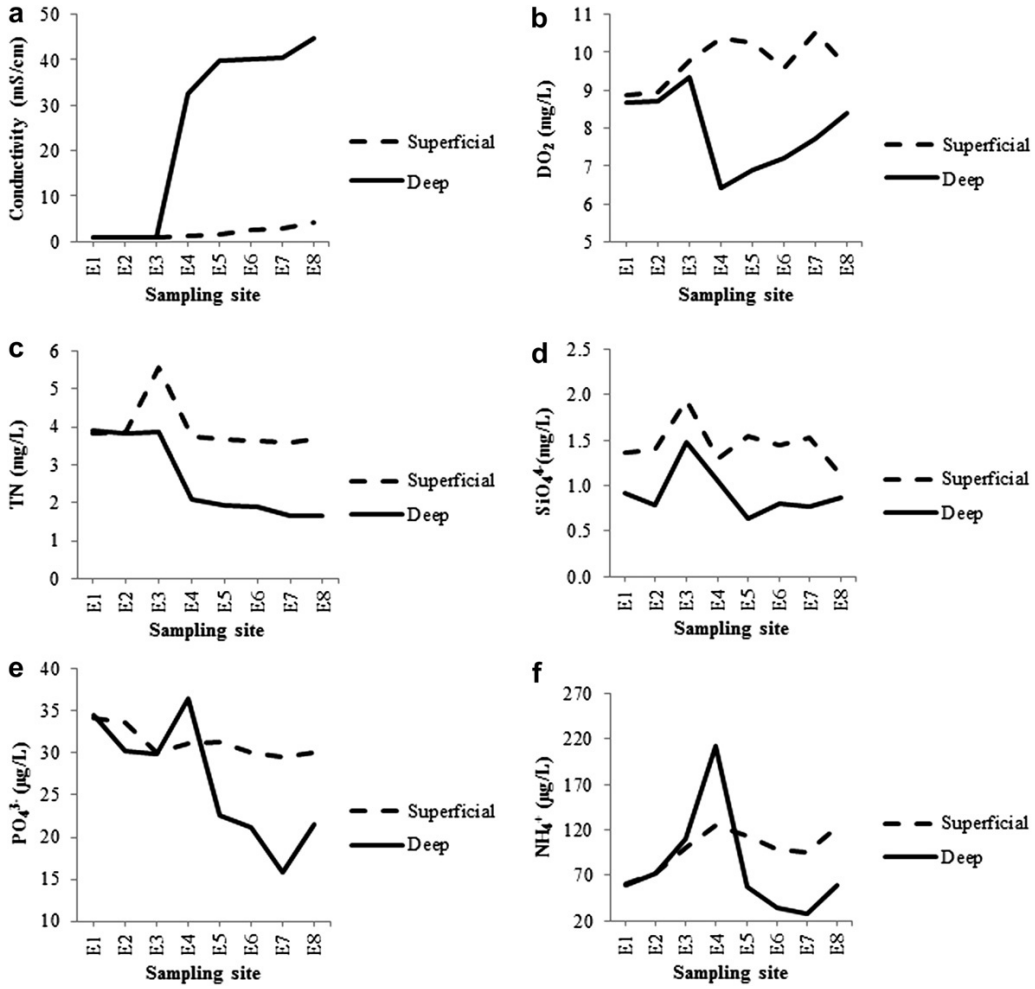

Fig. 2. Physicochemical parameters that accounted for spatial variability in the Ebro Estuary. SiO_4^{4-} = mg $Si{-}SiO_4^{4-}$/L, PO_4^{3-} = µg $P{-}PO_4^{3-}$/L, NH_4^+ = µg $N{-}NH_4^+$/L.

Table 3
Diatom taxa with relative abundance (RA >0.2%) and present in more than 5% of the Ebro Estuary samples.

Diatom taxa	% RA	Diatom taxa	% RA
Achnanthes amoena Hustedt	0.80	*Navicula* aff. *normaloides* Cholnoky	0.20
Achnanthidium minutissimum (Kützing) Czarnecki	1.42	*Navicula perminuta* Grunow	1.13
Achnanthes sp.	0.28	*Navicula* cf. *perminuta* Grunow	1.25
Amphora inariensis Krammer	0.29	*Navicula recens* (Lange-Bertalot) Lange-Bertalot	2.36
Amphora indistincta Levkov	2.22	*Navicula* aff. *recens* (Lange-Bertalot) Lange-Bertalot	0.27
Amphora aff. *luciae* Cholnoky	0.49	*Navicula reichardtiana* Lange-Bertalot	0.20
Amphora cf. *meridionalis* Levkov	0.61	*Navicula tripunctata* (O.F. Müller) Bory	0.36
Amphora ovalis (Kützing) Kützing	0.21	*Navicula veneta* Kützing	0.22
Amphora pediculus (Kützing) Grunow	8.32	*Nitzschia amphibia* Grunow	0.40
Amphora polita Krasske	0.58	*Nitzschia constricta* (Kützing) Ralfs	0.35
Amphora cf. *vetula* Levkov	1.83	*Nitzschia dissipata* (Kützing) Grunow	2.20
Bacillaria paxillifera (O.F. Müller) Hendey	2.57	*Nitzschia filiformis* (W. Smith) Van Heurck	0.90
Cocconeis cf. neothumensis var. marina De Stefano, Marino & Mazzella	0.71	*Nitzschia* cf. *fonticola* (Grunow) Grunow	0.41
Cocconeis pediculus Ehrenberg	1.03	*Nitzschia frustulum*[*] (Kützing) Grunow	8.19
Cocconeis placentula var. *euglypta* (Ehrenberg) Grunow	14.62	*Nitzschia frustulum* var. *bulnheimiana* (Rabenhorst) Grunow	0.81
Cocconeis placentula var. *placentula* Ehrenberg	1.34	*Nitzschia inconspicua* Grunow	5.44
Cocconeis placentula var. *trilineata* (M. Peragallo & J. Héribaud) Cleve	5.30	*Nitzschia microcephala* Grunow	0.21
Cyclotella meneghiniana Kützing	0.26	*Nitzschia palea* (Kützing) W. Smith	0.74
Diploneis sp.	1.15	*Nitzschia* cf. *palea* (Kützing) W. Smith	0.60
Eolimna subminuscula (Manguin) Moser, Lange-Bertalot & Metzeltin	0.43	*Nitzschia prolongata* Hustedt	0.34
Fallacia clepsidroides Witkowski	0.41	*Nitzschia* cf. *sociabilis* Hustedt	0.24
Gomphonema grovei var. *lingulatum* (Hustedt) Lange-Bertalot	0.40	*Parlibellus* cf. *berkeleyi* (Kützing) Cox	0.23
Gomphonema cf. *minutum* (Agardh) Agardh	0.25	*Planothidium iberense* Rovira & Witkowski	0.43
Gomphonemopsis obscura (Krasske) Lange-Bertalot	0.30	*Pleurosira laevis* (Ehrenberg) Compère	0.24
Gomphonema parvulum (Kützing) Kützing	0.22	*Psammothidium punctulatum* (Simonsen) Bukhtiyarova et Round	0.30
Melosira varians Agardh	0.35	*Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot	7.24
Navicula antonii Lange-Bertalot	1.21	*Synedra ulna* (Nitzsch) Ehrenberg	0.21
Navicula capitatoradiata Germain	0.21	*Tabularia fasciculata* (Agardh) Williams & Round	2.67
Navicula cryptotenella Lange-Bertalot	2.52	*Tabularia tabulata* (Agardh) Snoeijs	1.59
Navicula cf. *cryptotenelloides* Lange-Bertalot	0.59	*Thalassiosira pseudonana* Hasle & Heimdal	0.22
Navicula gregaria Donkin	0.54		
Navicula aff. *mollis* (W. Smith) Cleve	0.72		

[*] *Nitzschia frustulum* sensu lato: Our recent investigation suggests that *N. frustulum* specimens identified here correspond to long cells of *N. inconspicua* after auxosporulation. Though, we still maintain the division between the two taxa since they are still identified as two different entities in recent literature.

column (except the December campaign, with a well-mixed water column due to high river flows). Following the same pattern than clusters 1 and 2, October 2007 downstream samples were concentrated in cluster 3 while April 2008 samples were assembled in cluster 4. The SIMPER analysis showed that clusters 1 and 2 (mostly upstream sites) presented higher intragroup similarity than clusters 3 and 4 (containing downstream sites). *Cocconeis placentula* var. *euglypta* was the most contributing species characterising clusters 1(21.13%) and 3 (10.59%), whereas *Amphora pediculus* (Kützing) Grunow was the most contributing species in cluster 2 (16.23%) and *Nitzschia frustulum* (Kützing) Grunow in cluster 4 (22.7%) (Table 5; Fig. 4).

The DCA first two axes accounted for 23.9% of total variation of diatom communities (Fig. 5). The first axis (16.2% of the variation) differentiated diatom assemblages from downstream sites (clusters 3 and 4) from those mainly from upstream ones (clusters 1 and 2). The second axis (7.7% of the variation) arranged diatom assemblages on an apparently seasonal basis, grouping those of October from those of April (i.e. cluster 1 and 3 from clusters 2 and 4), while the rest of sampling campaigns remained scattered within clusters. The DCA ordination results supported the cluster division of sites according to diatom composition.

3.3. Environmental factors affecting diatom assemblages

CVA with forward selection of environmental data (Table 6) indicated that 'distance from the sea', Ebro River flow fluctuation, water chlorophyll *a* and pH were the environmental variables that accounted for significant portions of the total variance in diatom assemblages (i.e. established clusters) (28.2% by the first two axes, $P < 0.01$). Distance from the sea explained the largest portion (15.3%) of the total unconstrained variance while pH, Ebro River flow fluctuation and phytoplankton chlorophyll *a* explained 6%, 4.4% and 2.8% respectively.

The CVA first axis (18.8% of total variance of data) correlated well and negatively with 'distance from the sea' variable ($r = -0.67$, $P < 0.05$). Distance from the sea was in turn negatively correlated with conductivity and positively with TP (Table 7), and similarly the DCA first axis separated diatom assemblages from estuarine conditions (sites with marine influence at both superficial and deep water layers, i.e. clusters 3 and 4) from those of riverine conditions (mostly sites with no or weak marine influence, as is the case of clusters 1 and 2). Thus, this axis could be related to a marine influence gradient. The second CVA axis (9.4% of total variation in species data) was correlated with pH ($r = -0.34$, $P < 0.05$), Ebro River flow fluctuations ($r = 0.43$, $P < 0.05$) and phytoplankton chlorophyll *a* ($r = -0.31$, $P < 0.05$) and arranged diatom assemblages on an apparently seasonal basis, grouping those of October (clusters 1 and 3) from those of April (clusters 2 and 4). Spearman correlations (Table 7) showed that pH was positively correlated with temperature and negatively with $N{-}NO_2^-$, $N{-}NO_3^-$ and TN, whereas water chlorophyll *a* was positively correlated with particulate organic matter (POM) and negatively with $P{-}PO_4^{3-}$, $N{-}NO_3^-$ and TN, and Ebro River flow fluctuation was negatively correlated with conductivity, water chl *a*, $Si{-}SiO_4^{4-}$ and POM, and positively with TSS and all nitrogen compounds.

As riverine and estuarine conditions have different hydrological dynamics, their diatom assemblages were analysed separately. Therefore, clusters 1 and 2 (diatom assemblages of riverine conditions) and clusters 3 and 4 (diatom assemblages of estuarine conditions) were related to all the environmental data with two independent RDA sets (Figs. 6 and 7). In the RDA of riverine conditions, both axes explained 19.6% of total diatom variance and 88.8% of explained species-environmental variance (Fig. 6). The first

Table 4
Mean, minimum, maximum and coefficient of variation (CV) of physicochemical parameters of each diatom assemblage (cluster). The mean of pH was calculated as the mean of [H+] and converted back to a pH value afterwards.

Cluster	Temperature (°C)				pH				DO_2 (mg/L)				Conductivity (mS/cm)				$P-PO_4^{3-}$ (µg/L)				TP (µg/L)				$N-NH_4^+$ (µg/L)			
	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV
1	20.2	11.0	25.2	27	8.17	7.88	8.37	1	8.8	6.9	17.7	30	1.26	0.75	2.92	42	32.8	7.7	57.7	47	59.9	31.1	103.0	27	64.9	7.0	126.9	38
2	16.4	10.7	25.2	31	8.03	7.84	8.34	2	9.5	5.5	19.1	30	3.05	0.76	52.57	324	32.3	7.7	52.9	56	51.4	26.3	93.5	45	114.6	9.6	472.5	104
3	17.6	10.8	23.7	33	8.12	7.95	8.35	2	10.5	2.2	20.5	54	16.08	0.97	52.25	136	32.4	6.2	62.9	50	58.3	20.0	114.6	42	74.4	17.1	294.8	86
4	17.3	10.8	25.1	27	8.0	7.72	8.30	2	8.6	4.8	15.6	26	11.91	0.97	43.05	137	26.8	3.8	56.0	53	46.9	14.6	93.9	36	176.1	23.2	720.6	105

Cluster	$Si-SiO_4^{4-}$ (mg/L)				Water chl *a* (µg/L)				TSS (mg/L)				POM (%)				$N-NO_3^-$ (mg/L)				$N-NO_2^-$ (µg/L)				TN (mg/L)			
	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV	Mean	Min	Max	CV
1	1.4	0.6	2.9	46	0.97	0.45	2.88	66	3.69	0.98	8.21	53	40.07	14.29	89.13	54	2.34	1.51	3.98	38	14.3	4.2	22.2	35	3.3	2.0	7.1	51
2	1.3	0.3	2.9	53	1.06	0.39	5.08	99	5.96	0.36	22.10	80	27.22	0.36	49.80	40	3.13	0.04	4.62	36	25.1	5.2	55.5	84	4.5	0.2	6.7	68
3	1.1	0.1	2.5	60	1.86	0.49	4.07	64	4.32	2.05	17.11	89	33.22	12.31	59.96	45	2.00	0.03	3.88	74	15.0	0.3	23.3	46	2.7	0.1	6.7	75
4	1.2	0.1	2.1	35	0.90	0.28	2.86	67	4.83	2.05	17.08	77	29.30	12.31	66.18	38	2.89	0.09	4.28	44	28.6	6.3	55.5	56	3.9	0.1	6.3	44

RDA axis (15.2% of total species variation and 69.1% of fitted variation) was highly and negatively correlated to pH ($r = -0.63$, $P < 0.05$) and clearly differentiated January and April diatom assemblages (low pH values and low minimum river flow) from those of the rest of sampling campaigns (mainly with higher pH values and higher minimum river flow).Thus, this axis is differentiating diatom assemblages of riverine conditions under low flows for a long period of time (i.e. January and April) from the diatom assemblages established after high flows (gt; 400 $m^3\ s^{-1}$) that occurred after natural rainfall periods both in spring 2007 and 2008 and in late autumn 2008.

The second RDA axis (4.4% of total diatom variance, 19.7% of explained variance) was positively related to Ebro River flow fluctuations ($r = 0.63$, $P < 0.05$) and water chlorophyll *a* ($r = 0.19$, $P < 0.05$). Although this axis explained a low portion of total variance it is possible to relate it to a secondary response of diatom community to dynamic and fluctuating conditions due to Ebro River flow fluctuations. This effect is more noticeable comparing extreme situations, i.e. January 2008 (without fluctuations for a long period) and July 2008 (sudden decrease in river flow after strong floods in May 2008).

For diatom assemblages of estuarine conditions (clusters 3 and 4), the first two RDA axes explained 20.3% of total variance and 57.2% of explained variance. The first RDA axis (11.4% of total variance, 32.2% of explained variance) was well and positively correlated to fluctuations in Ebro River flow ($r = 0.54$, $P < 0.05$) but also negatively to 'distance from the sea' ($r = -0.38$, $P < 0.05$) and pH ($r = -0.42$, $P < 0.05$) (Fig. 7a). This axis seems to represent once more the response of diatom community to dynamic and fluctuating conditions but in a temporal and spatial scale. Situations with low river flow fluctuation and therefore a well-established salt-wedge (i.e. October, January and September) were grouped in front of fluctuating conditions where the salt-wedge is disappearing (April), has disappeared (December) or has recently penetrated into river channel after spring floods (July). Due to the gaps of data in July and December, the diatom dynamics are more difficult to fully interpret, although it seems that July diatom assemblages and at least half of December diatom assemblages were also related to fluctuating conditions present in April. There is a secondary gradient in dynamic conditions at spatial scale, although it is more subtle to observe due to the lack of samples in sites close to the sea. Diatom assemblages in sites close to the sea respond to more mixing and unstable conditions than upstream diatom assemblages.

The second estuarine RDA axis (8% of total variance and 25.9% of explained variance) was correlated positively to conductivity ($r = 0.63$, $P < 0.05$), $P-PO_4^{3-}$ concentration ($r = 0.24$, $P < 0.05$) and negatively to pH ($r = -0.42$, $P < 0.05$) (Fig. 7b). This axis is likely to represent a marine influence due to the presence and persistence of the salt-wedge (with higher conductivity and lower nutrients, irradiance, water velocity, pH values and temperature), and therefore disturbing the diatom community from deep water layers when the salt-wedge was present as well as sites close to the sea at superficial water layers. The positive correlation with $P-PO_4^{3-}$ could be related to the limit of the salt-wedge (E4D) in stable situations with no river flow fluctuations (September and October) (Fig. 2e).

4. Discussion

4.1. Physicochemical conditions in the Ebro Estuary

In the last few decades, the presence of the salt-wedge in the Ebro Estuary has become more regular and frequent due to the flow reduction and regulation of the Ebro River (Ibáñez et al., 1996;

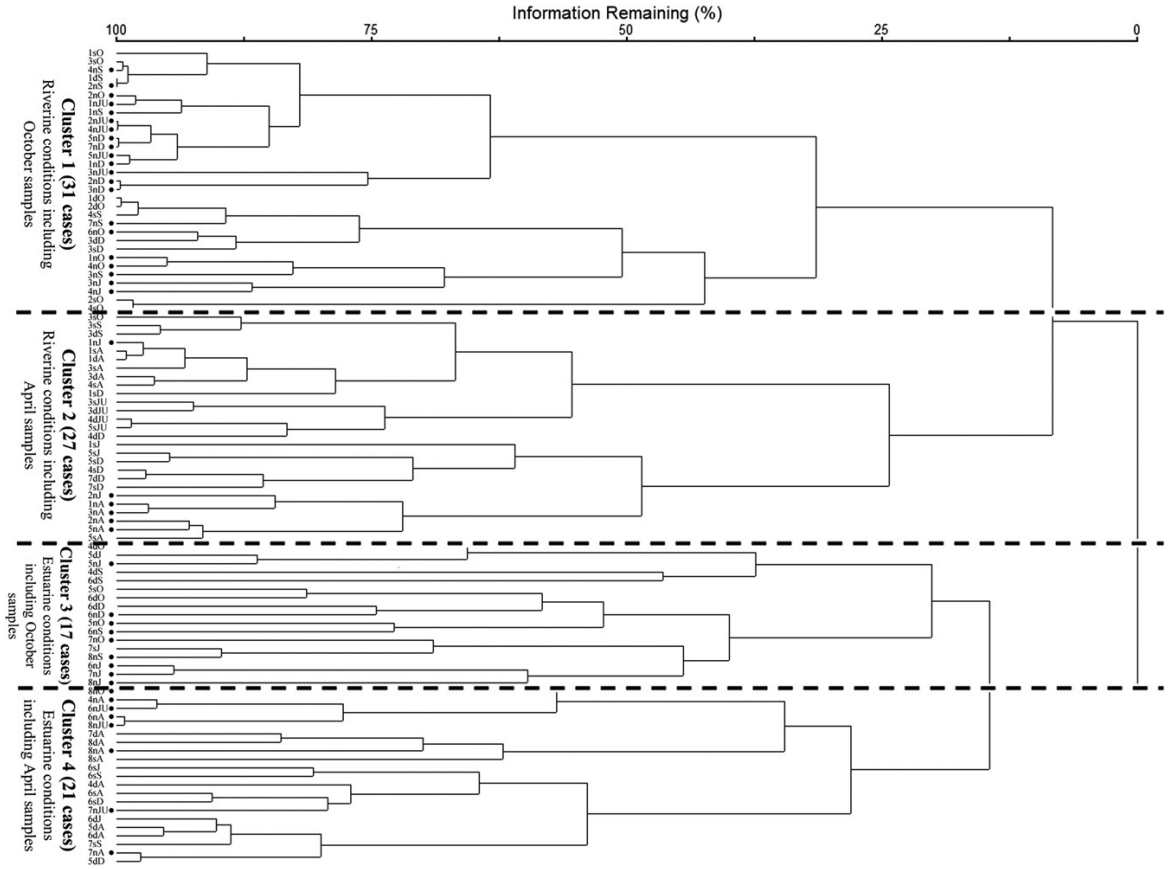

Fig. 3. Cluster dendogram of the 96 samples according to similarity in diatom composition. s- superficial, d- deep, dark circles: natural substrata. O = October 2007, J = January 2008, A = April 2008, JU = July 2008, S = September 2008, D = December 2008.

Sierra et al., 2004). The increased marine influence in the Ebro Estuary implied not only the presence of the salt-wedge at deep water layers during most of the sampling campaigns but also, at a smaller scale, a stronger marine influence at the superficial water layers in sites close to the sea due to the advective circulation of the salt-wedge and the wind-induced sea water mixing. This more regular and stable marine influence causes the establishment of two contrasting conditions in the Ebro Estuary: 1) a riverine condition which usually occupies the upper 14 km of the estuary with a rather homogenous water column due to the fact that seawater intrusion hardly occurs; 2) an estuarine condition (which most of the year comprises the last 18 km of the estuary), where the marine influence affects both superficial and deep water layers. The limit of riverine and estuarine conditions depends on the salt-wedge position and can be altered especially by moments of high or low river flow. In the first case the marine influence disappears and therefore the whole estuary resembles a "riverine" situation, while the opposite situation happens in the second case.

4.2. Environmental factors affecting diatom community

The hydrological dynamics of the Ebro Estuary affected benthic diatom community both at spatial and temporal scale. As a result of the complex dynamics involving the Ebro River and the sea, the marine influence appeared as the main factor affecting spatial diatom composition and distribution when all Ebro Estuary samples are considered. The marine influence is represented by the 'distance from the sea' and is well correlated with conductivity and with water phosphorous concentration. Some previous studies in estuaries and similar systems (McIntire, 1978; Admiraal, 1984; Laird and Edgar, 1992; Underwood, 1994; Peletier, 1996) pointed out that salinity has a key role in the diatom community composition and distribution. However, in such fluctuating systems, the salinity gradient often co-varies with nutrient concentrations, and it is not possible to separate the effects of each parameter (Underwood et al., 1998; Underwood and Provot, 2000; Trobajo et al., 2004a). The present study showed that salinity (or conductivity) solely cannot be considered as a driving force determining diatom species composition and distribution in this system, although conductivity can be considered as a surrogate of the marine influence. The latter also includes other physicochemical gradients that co-vary with conductivity, e.g. the decrease in phosphorous concentration, making very difficult to disentangle the effect due to each of them.

Interestingly, a vertical marine influence was also identified in the diatom assemblages of estuarine conditions. Deep water layers with salt-wedge presence are not only affected by high conductivities and low nutrient concentration, but also by lower irradiances and lower dissolved oxygen due to the higher water residence time (Casamayor et al., 2001; Cruzado et al., 2002; Rovira et al., 2009). The minimum oxygen values in addition with high peaks of total phosphorous concentration and ammonium were

Table 5
SIMPER analyses – diatom species that contributed for each cluster similarity. SIM (%) represents percentage of contribution of individual species to intragroup similarity.

Cluster 1 - riverine		Cluster 2 - riverine		Cluster 3 - estuarine		Cluster 4 - estuarine	
Simper species	SIM (%)	Simper species	SIM (%)	Simper species	SIM (%)	Simper species	SIM (%)
Cocconeis placentula var. *euglypta*	21.13	*Amphora pediculus*	16.23	*Cocconeis placentula* var. *euglypta*	10.59	*Nitzschia frustulum*	22.70
Cocconeis placentula var. *trilineata*	12.13	*Cocconeis placentula* var. *euglypta*	8.73	*Tabularia fasciculata*	9.48	*Nitzschia inconspicua*	11.48
Navicula cryptotenella	5.64	*Rhoicosphenia abbreviata*	8.20	*Navicula recens*	8.08	*Rhoicosphenia abbreviata*	9.20
Amphora pediculus	5.64	*Amphora indistincta*	6.71	*Rhoicosphenia abbreviata*	7.59	*Cocconeis placentula* var. *euglypta*	5.69
Rhoicosphenia abbreviata	5.18	*Nitzschia inconspicua*	6.15	*Bacillaria paxillifera*	4.74	*Navicula recens*	4.22
Navicula antonii	4.51	*Cocconeis placentula* var. *trilineata*	4.76	*Nitzschia frustulum*	4.32	*Amphora pediculus*	3.75
Navicula recens	4.02	*Navicula cryptotenella*	4.73	*Amphora pediculus*	3.96	*Tabularia fasciculata*	3.16
Bacillaria paxillifera	3.84	*Nitzschia frustulum*	4.42	*Navicula* aff. *mollis*	3.39	*Navicula perminuta*	2.83
Tabularia fasciculata	3.15	*Achnanthidium minutissimum*	4.33	*Tabularia tabulata*	3.26	*Navicula* aff. *mollis*	2.29
Cocconeis pediculus	2.52	*Navicula antonii*	3.69	*Nitzschia inconspicua*	3.16	*Cocconeis placentula* var. *trilineata*	2.22
Nitzschia dissipata	2.34	*Nitzschia dissipata*	3.57	*Navicula cryptotenella*	2.96	*Navicula gregaria*	2.09
Amphora cf. *vetula*	2.33	*Amphora* cf. *meridionalis*	3.00	*Nitzschia dissipata*	2.83	*Amphora polita*	2.07
Nitzschia palea	2.30	*Amphora* cf. *vetula*	2.89	*Nitzschia filiformis*	2.82	*Navicula cryptotenella*	2.05
Nitzschia inconspicua	2.09	*Cocconeis pediculus*	2.21	*Cocconeis placentula* var. *trilineata*	2.65	*Nitzschia dissipata*	1.68
Nitzschia frustulum	2.07	*Nitzschia frustulum* var. *bulnheimiana*	1.93	*Navicula antonii*	2.55	*Bacillaria paxillifera*	1.67
Cocconeis placentula var. *placentula*	1.97	*Bacillaria paxillifera*	1.84	*Navicula* cf. *perminuta*	1.59	*Nitzschia filiformis*	1.66
Nitzschia amphibia	1.82	*Cocconeis placentula* var. *placentula*	1.56	*Nitzschia constricta*	1.59	*Nizschia* cf. *palea*	1.18
Navicula cf. *cryptotenelloides*	1.82	*Nitzschia* cf. *fonticola*	1.38	*Nitzschia palea*	1.47	*Navicula veneta*	1.18
Achnanthidium minutissimum	1.44	*Navicula recens*	1.38	*Nitzschia* cf. *palea*	1.36	*Amphora indistincta*	1.15
Navicula tripunctata	1.41	*Nitzschia amphibia*	1.38	*Eolimna subminuscula*	1.28	*Amphora* cf. *meridionalis*	1.14
Navicula capitatoradiata	1.39	*Tabularia fasciculata*	1.36	*Navicula perminuta*	1.27	*Tabularia tabulata*	1.10
Nitzschia filiformis	1.31	**Total**	**90.45**	*Amphora* cf. *vetula*	1.19	*Achnanthes* sp.	1.09
Total	**90.05**	**Average similarity**	**55.58**	*Navicula gregaria*	1.14	*Diploneis* sp.	0.98
Average similarity	**59.03**			*Amphora polita*	1.06	*Navicula antonii*	0.91
				Navicula tripunctata	1.02	*Amphora* cf. *vetula*	0.90
				Cocconeis pediculus	1.00	*Cocconeis* cf. *neothumensis* var. *marina*	0.87
				Navicula cf. *recens*	0.99	*Nitzschia constricta*	0.79
				Diploneis sp.	0.97	**Total**	**90.05**
				Nitzschia prolongata	0.96	**Average similarity**	**46.74**
				Pleurosira laevis	0.76		
				Total	**90.03**		
				Average similarity	**42.22**		

associated with the conditions near the limit of the salt-wedge (usually E4D-E5D), where the water can remain still for a long time and this could increase the deposition of nutrients and denitrification processes, with the consequent oxygen consumption by biological activity (Ibáñez et al., 1995; Casamayor et al., 2001). A special case was found in E4D in July 2008, which showed relatively high water chlorophyll *a* values (5 μg l^{-1}). The maximum water chlorophyll *a* value (14.5 μg l^{-1}) was found at E5D also in July. High temperatures and the sudden decrease of the Ebro River flow (from gt; 400 m^3 s^{-1} to <100 m^3 s^{-1} in 10 days) caused the sudden intrusion of the salt-wedge that could have promoted the accumulation of dying phytoplankton cells near the salt-wedge limit.

The benthic diatom community of the Ebro Estuary seems to respond seasonally to the hydrological regime of Ebro River flows. However, it is not likely that seasonality alone determined diatom distribution and thus community composition, as has already been suggested in other studies on similar systems (Underwood and Kromkamp, 1999; Underwood and Provot, 2000; Trobajo et al., 2004a). Temporal variability involves different processes (e.g. the increase of floods, sudden river flow fluctuations, changes in light and temperature, differences in nutrient concentrations) and therefore it is the occurrence and interaction of some or all these factors at certain times of the year that affects the diatom composition and distribution. The temporal variability in diatom communities of the Ebro Estuary seems to be very closely related to both the Ebro River flow fluctuations and minimum flow levels for a long period, supporting studies pointing the importance of hydrological processes when studying diatom communities (Martínez de Fabricius et al., 2003; Wiklund et al., 2010), especially in estuarine conditions where sudden floods or droughts produce a strong variability in physicochemical characteristics (Cloern et al., 1983; Trigueros and Orive, 2000; Paerl et al., 2006).

Even though October and April diatom samples remain divided both in riverine and estuarine conditions, the environmental processes that force their distinction are different when analysing each condition separately. Diatom assemblages under riverine conditions rarely revealed a marine influence, and they inhabit more stable conditions due to the less fluctuating dynamics. In the absence of intrusions or disappearances of the salt-wedge, the diatom community is mainly affected by seasonal variability in pH and long periods of low flows. Periods of rainfall occurred during spring (February–April) and therefore upstream reservoirs recovered their maximum capacity with renewed water. The opening of reservoir floodgates at the end of April 2007 and beginning of May 2008 caused a sudden increase of river flow (900–1000 m^3 s^{-1}) for approximately 15 days. These high river flows seem to create a shift in diatom

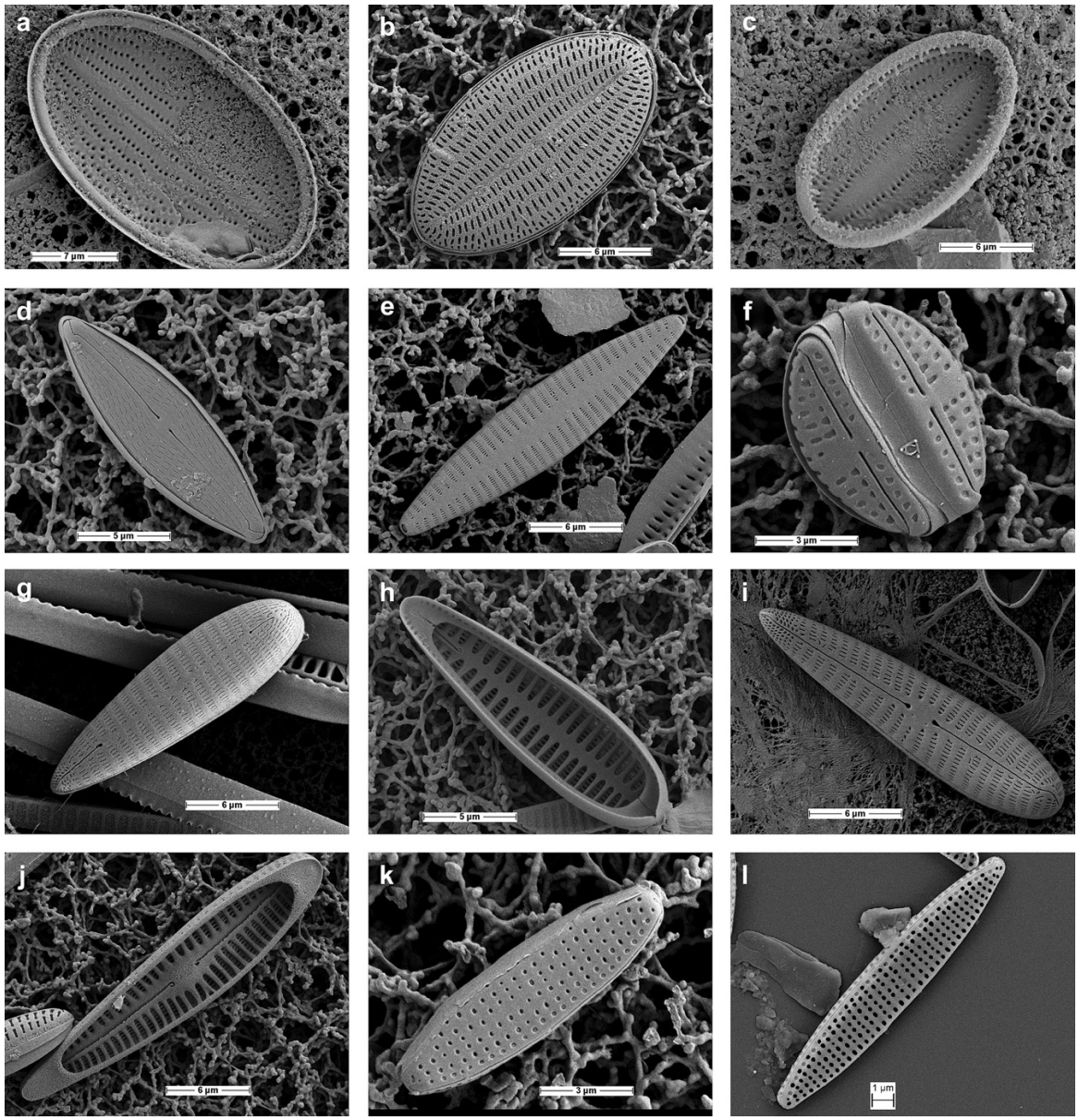

Fig. 4. Representative taxa in each diatom assemblage (cluster) under the SEM. a–b: *Cocconeis placentula* var. *euglypta* (rapheless valve); c: *Cocconeis placentula* var. *trilineata* (rapheless valve, interior view); d: *Navicula cryptotenella* (external view); e: *Tabularia fasciculata* (external view); f: *Amphora pediculus* (external view); g–j: *Rhoicosphenia abbreviata* (g–h: rapheless valve; i–j: raphe valve); k: *Nitzschia inconspicua* (external view); l: *Nitzschia frustulum* (external view).

community grouping summer and autumn conditions (after floods) in front of January and April conditions (before floods). This temporal variability in the diatom community seems to have an interannual variability depending on flow regime, as previously recorded in other estuarine systems (Cloern and Nichols, 1985; Guinder et al., 2010). In the present study, this interannual variability grouped December 2008 (under natural high flows after a rainfall period) with summer and autumn samples instead of being aggregated to January 2008 according to their similar temperature.

In contrast, under estuarine conditions the effect of the fluctuating dynamics of the Ebro River flow in diatom assemblages becomes more important because of the consequent intrusion or extrusion of the salt-wedge. In contrast to diatom assemblages of riverine conditions, diatom assemblages of estuarine conditions respond rapidly to fluctuations in river flow. Stable conditions with a well-established salt-wedge (January, September and October samples) remain divided from unstable and dynamic conditions with a salt-wedge fluctuation (April, July and December samples). Dynamic conditions will also have a spatial gradient, with upstream waters more stable and productive than assemblages close to the sea, as observed in phytoplankton communities from other estuarine systems (Revilla et al., 2000).

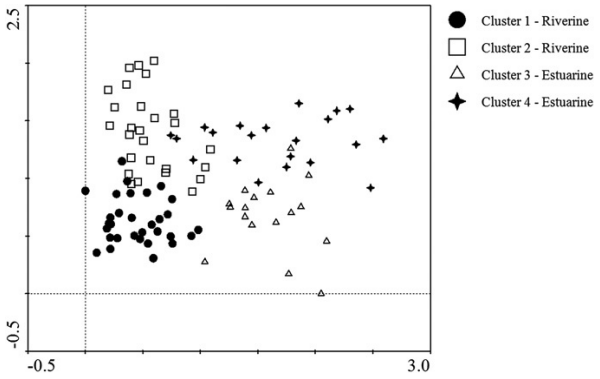

Fig. 5. DCA of diatom community in the Ebro Estuary. Riverine samples with few salt-wedge intrusions are represented by Cluster 1 (most of October 2007 samples) and Cluster 2 (most of April 2008 samples). Estuarine samples with marine influence both at superficial and deep layers are represented by Cluster 3 (most of October 2007 samples) and Cluster 4 (most of April 2008 samples).

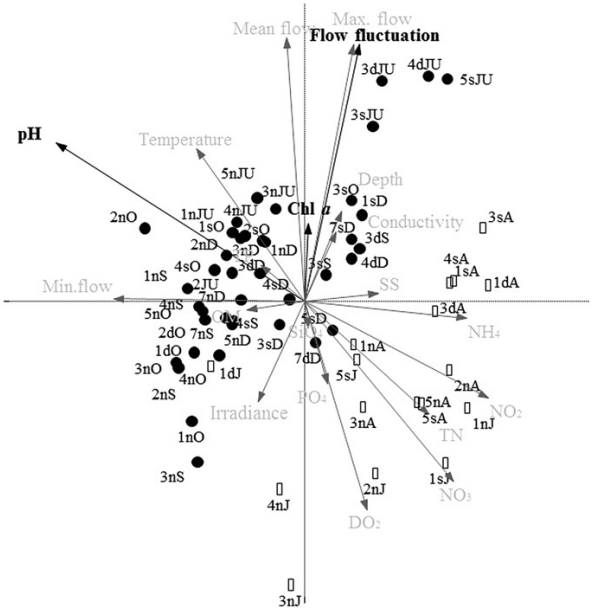

Fig. 6. RDA showing the relationship between diatom assemblages under riverine conditions and selected environmental variables. Significant environmental variables are displayed in black meanwhile supplementary variables have been added in light grey. Samples are shown by symbols according to the first axis. "E" label in sampling sites have been omitted. s- superficial, d- deep, n- natural. Min.flow = minimum Ebro River flow, Max.flow = maximum Ebro River flow. O = October 2007, J = January 2008, A = April 2008, JU = July 2008, S = September 2008, D = December 2008.

4.3. Ecological preferences of diatom assemblages

4.3.1. Spatial diatom assemblages

The diatom community composition reflected the distinction of riverine and estuarine conditions in the Ebro Estuary. Clusters 1 and 2 correspond to diatom assemblage characteristic of riverine conditions, typical of oligohaline waters, with minor conductivity fluctuations, and very few sudden salt-wedge intrusions. The diatom community is rather similar within sites, being *Cocconeis placentula* var. *euglypta*, *C. placentula* var. *trilineata*, *Navicula cryptotenella* and *Amphora pediculus*, widespread diatom species that thrive well in this riverine state. In contrast, clusters 3 and 4 correspond to diatom assemblages characteristic of estuarine conditions, with a marine influence at both superficial and deep water layers. This marine influence produces strong physichochemical gradients between sites, and as a consequence the diatom community reflects this variation being less similar within sites and constituted mainly of *Nitzschia frustulum, Nitzschia inconspicua*, *C. placentula* var. *euglypta*, *Tabularia fasciculata* and *Rhoicosphenia abbreviata*.

Despite the separation of the Ebro Estuary diatom community into riverine and estuarine assemblages, most abundant diatom species inhabit in both assemblages, with different proportions or combinations to produce the distinction between assemblages. Therefore, the abundance of some of these species depends on the prevailing ecological situation in the estuary. For example, high abundances of *Amphora pediculus* and *C. placentula* var. *euglypta* were more characteristic of riverine conditions, while high abundances of *N. frustulum* were mostly characteristic of estuarine situations. In contrast, there are diatom species that contributed only to riverine conditions (*C. placentula* var. *placentula*, *Nitzschia amphibia*, *Navicula* cf. *cryptotenelloides*, *Achnanthidium minutissimum*, *Navicula capitatoradiata*), while others were only characteristic of estuarine conditions (*Navicula* aff. *mollis*, *Diploneis* sp., *Navicula gregaria*, *Amphora polita*, *Nitzschia* cf. *palea*, *Navicula* cf. *perminuta*). These are type-specific taxa of riverine and estuarine situations, although they were present at low abundances and/or low frequencies. We can conclude that the most abundant and frequent diatom species in the Ebro Estuary can tolerate a wide range of conductivity and nutrient concentrations, especially in estuarine conditions, where there is high physicochemical variability between superficial and deep water layers due to water stratification. Our results reinforce other studies reporting this broad tolerance of diatom communities to changes in environmental variables in fluctuating systems (Underwood, 1994; Sullivan and Currin, 2000; Trobajo et al., 2004a; Bate and Smailes, 2008).

Table 6
Resume of the Canonical Variates Analyses (CVA) with forward selection of environmental variables.

	CVA axis		
	1	2	3
Eigenvalues	0.6	0.3	0.1
% total variance	18.8	28.2	31.1
% explained variance	60.6	90.9	100.0

Table 7
Spearman correlation coefficients between environmental variables statistically significant from CVA and the rest of physicochemical parameters. Only significant correlations ($P < 0.01$) are listed. All the environmental parameters except from pH have been logarithmically transformed.

	Temp (°C)	Conductivity (mS/cm)	$P{-}PO_4^{3-}$ (μg/L)	TP (μg/L)	$N{-}NH_4^+$ (μg/L)	$N{-}NO_2^-$ (μg/L)	$N{-}NO_3^-$ (mg/L)	TN (mg/L)	$Si{-}SiO_4^{4-}$ (mg/L)	Water chl *a* (μg/L)	POM (%)	TSS (mg/L)
pH	0.303					−0.632	−0.486	−0.299				
Water chl *a* (μg/L)			−0.296				−0.299	−0.327	0.391		0.391	
Sea distance (km)		−0.628		0.329								
Flow fluctuation (m^3/s)		−0.268			0.444	0.407	0.554	0.548	−0.537	−0.413	−0.537	0.534

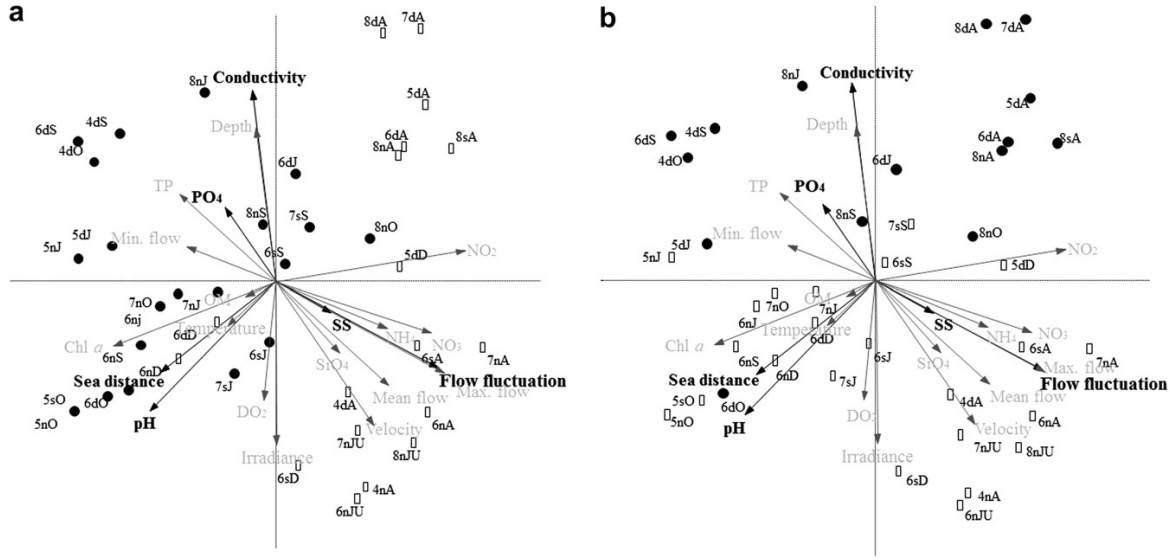

Fig. 7. RDA showing the relationship between diatom assemblages under estuarine conditions and selected environmental variables. Fig. 7a: Samples classified by symbols according to first axis. Fig 7b: Samples classified by symbols according to second axis. Significant environmental variables are displayed in black meanwhile supplementary variables have been added in light grey. "E" label in sampling sites have been omitted. s- superficial, d- deep, n- natural. Min.flow = minimum Ebro River flow, Max.flow = maximum Ebro River flow. O = October 2007, J = January 2008, A = April 2008, JU = July 2008, S = September 2008, D = December 2008.

It is of note that the benthic diatom community from the Ebro Estuary seems to be more affected by environmental parameters than by the substrata type, since no clear segregation was found between samples (Fig. 3, supplementary figure). Similar results were previously reported for the Ebro Estuary (Rovira et al., 2009) and for other ecosystems when comparing diatom assemblages from natural and artificial substrata (Lane et al., 2003), from several natural substrata (Winter and Duthie, 2000) and from different macroalgal hosts (Snoeijs, 1994).

4.3.2. Seasonal diatom assemblages

An increase in abundances of *Amphora pediculus*, *Amphora indistincta*, *Rhoicosphenia abbreviata*, and *Nitzschia inconspicua* characterised riverine conditions before the spring floods, and *Amphora pediculus* accounted for the highest abundance in these situations. While A. *pediculus* has been considered eutraphentic (Van Dam et al., 1994), other authors consider this species sensitive to organic pollution (Kwandrans et al., 1998). In the present study, this species was dominant in April but abundant also in other situations where suspended organic matter was still low. Therefore, it could be considered as a potential indicator of situations of low pH and low organic matter content.

In contrast, *Cocconeis placentula* var. *euglypta*, *C. placentula* var. *trilineata*, *Navicula cryptotenella* and *Navicula recens* abundances increased in riverine situations after spring floods, with higher temperatures and higher organic matter content. *C. placentula* var. *euglypta* also increased in sites close to the sea (E7) in the December campaign, where the Ebro River flow was high (gt; 500 $m^3 s^{-1}$) and therefore the salt-wedge disappeared approximately 10 days before sampling. This disturbance affected the diatom community and probably produced a rapid re-colonisation of available substrata patches in estuarine sites close to the sea (physically stressed areas due to the erosion of sea waves) by species more characteristic of riverine conditions (e.g. *C. placentula* var. *euglypta*). Ecological preferences of this diatom species are still not determined, being indicator of different salinity, nutrient and productivity ranges and hydrological conditions (Jones, 1978; Hill and Knight, 1988; Leland et al., 2001; Trobajo et al., 2004a; Gari and Corigliano, 2007; Licursi et al., 2010). In the present study, it occurred in a wide range of environmental conditions, indicating that it could represent an opportunistic and tolerant species that can rapidly colonise substrata (Hameed, 2003).

Diatom assemblages of estuarine conditions under well-established salt-wedge periods were characterised by *Cocconeis placentula var. euglypta*, *Tabularia fasciculata* and *Navicula recens*, but in dynamic situations they were dominated by *Nitzschia frustulum* and *Nitzschia inconspicua*. Both *N. frustulum* and *N. inconspicua* have been considered euryhaline species tolerant also to high organic matter and nutrient concentrations (Van Dam et al., 1994; Clavero et al., 2000; Ziemann et al., 2001; Trobajo et al., 2004a, 2004b). This broad tolerance of abundant and frequent estuarine diatom species, e.g. *N. frustulum* and *N. inconspicua*, has to be considered in further ecological studies, especially when assessing Ebro Estuary ecological status. In freshwater systems, where environmental strong fluctuations have been related to human disturbances, these species have been identified as species tolerant to pollution; while in the Ebro Estuary and other estuarine systems, this tolerance can be also due to natural environmental fluctuations (Dauvin, 2007; Elliott and Quintino, 2007).

The present study provides an ecological basis for further studies on temporal and spatial distribution of benthic diatom communities in highly stratified estuaries and similar ecosystems. In such heterogeneous and dynamic systems, the recognition of the ecological conditions that drive the benthic diatom community is of crucial importance as has been shown here. This study also shows the significance of the hydrological dynamics in affecting diatom community in a highly stratified estuary, where river flow magnitude and fluctuations create high physicochemical variability especially at sites close to the sea. Further studies on the effects of these hydrological processes on estuarine biota in general and diatoms in particular are required in order to success in assessing the Ebro Estuary ecological status according to European Water Framework Directive.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ecss.2011.12.033.

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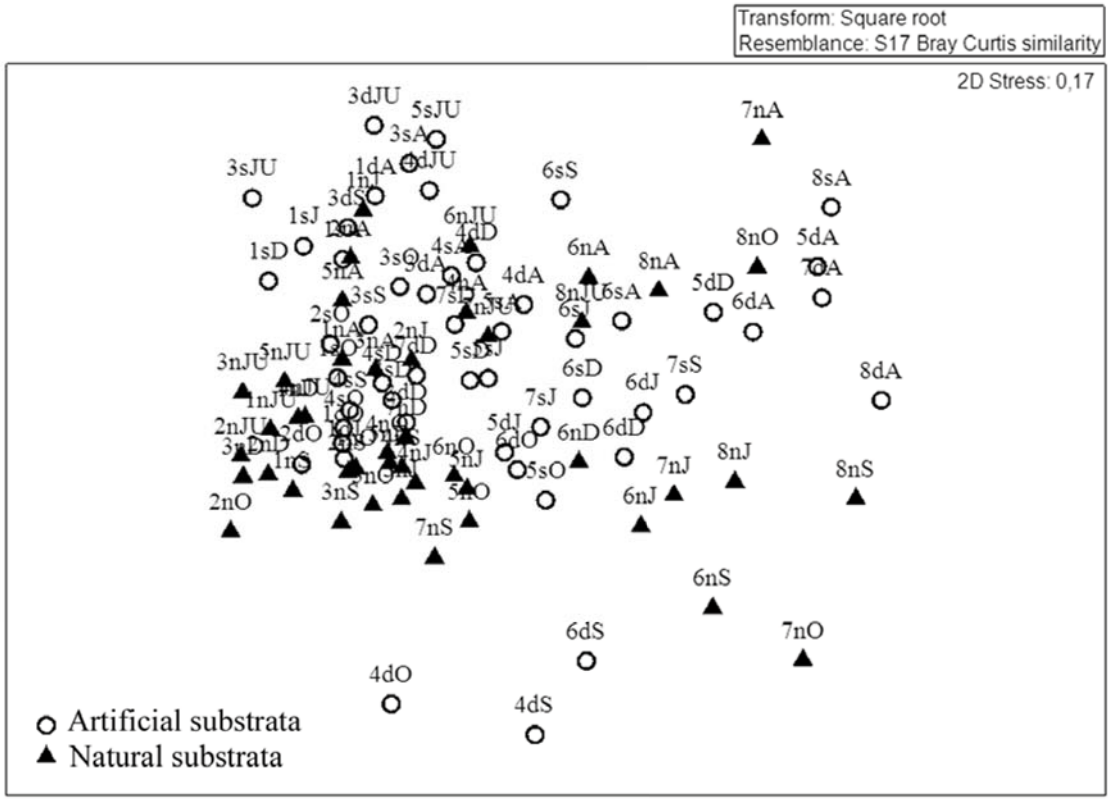

Fig. s1. NMDS based on similarity between samples using diatom species composition. s- superficial, d- deep, n- natural. O = October 2007, J = January 2008, A = April 2008, JU = July 2008, S = September 2008, D = December 2008.

Chapter 2

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The use of diatom assemblages as ecological indicators in highly stratified estuaries and evaluation of existing diatom indices

L. Rovira *, R. Trobajo, C. Ibáñez

IRTA – Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540 St. Carles de la Ràpita, Catalonia, Spain

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ABSTRACT

Diatom indices are used to evaluate the ecological status of rivers but they have been rarely applied in estuaries. This study aimed to identify the diatom species indicating the main environmental gradients and pressures in a highly stratified estuary; and to evaluate the applicability of existing freshwater diatom indices. Marine influence due to salt-wedge intrusion and sea water mixing appeared as the main factor affecting diatom community. Three diatom assemblages were identified: indicators of riverine conditions (without marine influence), indicators of estuarine conditions (heterogeneous conditions with higher conductivities due to marine influence) and those specifically indicating well-established salt-wedge situations. Nowadays, the main human pressure affecting diatom community in the Ebro Estuary is the hydrological alteration resulting from flow regulation and abstraction. Several limitations were encountered in the application of diatom indices (e.g. inverse response with nutrients; ecologically important species not considered). Therefore, their use in estuaries should be done cautiously.

1. Introduction

Estuaries and other transitional waters are dynamic ecosystems showing a high spatial and temporal physicochemical variability (Cloern et al., 1989; Rovira et al., 2009; Webster et al., 2000), and they can also present several pollution gradients due to the high number of human activities influencing them. Moreover, some natural stressors in transitional waters can be modified by human activities, making it very difficult to discern natural from anthropogenic stressors (Dauvin, 2007; Elliott and Quintino, 2007). Therefore, dominant estuarine flora and fauna will reflect this natural variability, but at the same time it may have features very similar to those found in anthropogenically stressed areas difficulting the detection of anthropogenic stress effects and, consequently, the accurate assessment of ecological status in estuaries (Elliott and Quintino, 2007).

The Water Framework Directive (WFD; EC, 2000) aims to assess the ecological status of all European water bodies (including transitional waters) using hydromorphological, physicochemical and biological indicators (i.e. phytoplankton, macroalgae, phytobenthos, macroinvertebrates and fish) (Allan et al., 2006; Borja et al., 2004; Logan and Furse, 2002). Due to its reduced mobility and short generation times, phytobenthos has shown a rapid response to environmental changes and can integrate environmental conditions better than other bioindicators (Smol and Stoermer, 2010); being commonly used in the assessment of the ecological status and monitoring of anthropogenic impacts. Diatoms are the main component of phytobenthos, being one of the most important algae groups used for ecological assessment (Descy and Coste, 1991; Kelly et al., 1995, 1998; King et al., 2006; Warwick et al., 1990). Their ubiquity, their direct and sensitive response to physicochemical changes, and their preservation in sediments for a long time makes them good water quality indicators for both present and past environmental changes (Smol and Stoermer, 2010).

In Europe there are about 20 diatom-based metrics that were initially developed to assess nutrient and/or organic pollution in rivers and, later, some of them have been adapted to fulfil the WFD requirements of assessing the ecological status of these ecosystems (Kelly et al., 2009). Nowadays, diatom indices are being routinely used in most EU member states to evaluate the ecological quality of rivers and streams. However, little information is available about the use of benthic diatoms as bioindicators in estuaries and other transitional systems, with only very few studies carried out in Europe (Bogaczewicz-Adamczak and Dziengo, 2003; Della Bella et al., 2007; Zgrundo and Bogaczewicz-Adamczak, 2004) and in USA (Bauer et al., 2007). The study of Della Bella et al. (2007) is the only one dealing with the controversies of water quality assessment in these complex water bodies.

The present study is the first attempt to evaluate the use of benthic diatoms to assess the ecological status in a highly stratified estuary. Highly stratified estuaries are usually characterised by weak tides, resulting in strong vertical water column stratification

* Corresponding author.
E-mail address: laia.rovira.torres@gmail.com (L. Rovira).

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that affects biological communities (Ibáñez et al., 1997; Nebra et al., 2011; Rovira et al., 2009). The study had two main objectives: the first was to identify the main environmental gradients in the Ebro Estuary and establish diatom indicator species associated with these gradients; the second objective was to identify the most relevant human pressures and evaluate the application of existing freshwater diatom indices to assess the ecological status of a highly stratified estuary, namely the Ebro Estuary.

2. Materials and methods

2.1. Study area

The Ebro Estuary covers an approximate area of 10 km^2 and is 40 km long with a mean width of 237 m and a mean depth of 6.8 m. It is considered a "micro-tidal salt-wedge estuary" with a tidal range around 20 cm. This weak tidal range favours the vertical stratification of the water column and the existence of a salt wedge, with a maximum intrusion in the Ebro River of 32 km. The hydrology and dynamics of this salt wedge are controlled mainly by the Ebro River flow, disappearing when the Ebro River flow is above 400 m^3/s. Between 250 and 400 m^3/s the salt wedge occupies the last 5 km of the estuary, and with discharges below 250 m^3/s, the salt wedge advances up to 18 km from the river mouth (this is the most frequent situation). When the river flow is less than 100 m^3/s, the salt wedge reaches its maximum extent (32 km from the river mouth), although this situation is much less frequent (Ibáñez et al., 1997). The lower Ebro River flow has been largely regulated since 1960s with two reservoirs (Mequinenza and Riba-Roja) situated 100 km upstream the river mouth for hydropower purposes, and it has decreased by 40% due to intensive water uses in the Ebro basin, with irrigation accounting for 90% of water consumed (Ibáñez and Prat, 2003).

2.2. Sampling

Eight sampling sites distributed every 3–6 km within the estuary were sampled every 3 months from October 2007 to December 2008 (Fig. 1). Benthic diatom samples were collected from both natural substrata (mainly macrophytes *Potamogeton pectinatus* and *Ceratophyllum* spp., and wood debris where macrophytes were not available) and artificial substrata (fired clay bricks). Fired clay bricks were placed at superficial (0.5 m) and deep (4–6 m) levels at each site. This sampling design allowed the gathering of both vertical and horizontal physicochemical gradients in the estuary. Artificial substrata were considered robust enough to resist high flows and sudden flow fluctuations that characterise the lower Ebro River. However, due to sudden increases in the river flow, some samples of artificial substrata were not recovered during the sampling period. An area of 4 cm^2 was scrapped off the artificial substrata and three fragments from natural substrata were included in each replicate. Two replicates from both artificial and natural substrata were processed in each site.

Water depth, temperature, electrical conductivity (EC_{25}), dissolved oxygen (DO_2), and pH were measured *in situ* using an YSI 556 multiprobe. Flow direction and velocity were also measured *in situ* using a BFM 001 current flow meter.

Lower Ebro River flow records (measured in Tortosa, 40 km upstream from the river mouth) were obtained from the Ebro Hydrographical Confederation (CHE) database. Historical data from 1913 to 2008 was used to analyse the annual average of daily flow and monthly fluctuation range (difference between maximum and minimum flow during 1 month) of lower Ebro River flow. The data from 30 days before sampling was used to calculate average, maximum, minimum, and fluctuation range of the lower Ebro River flow during each sampling campaign.

2.3. Nutrient and chlorophyll analyses

Water samples were collected both at superficial and deep water layers in order to determine nutrients and chlorophyll *a* content. Dissolved nutrients: silicate (Si-SiO_4^{4-}), nitrate (N-NO_3^-), nitrite (N-NO_2^-), phosphate (P-PO_4^{3-}), ammonium (N-NH_4^+); total nitrogen (TN) and total phosphorus (TP) were measured following Koroleff et al. (1977). Historical data for phosphate (P-PO_4^{3-}) and

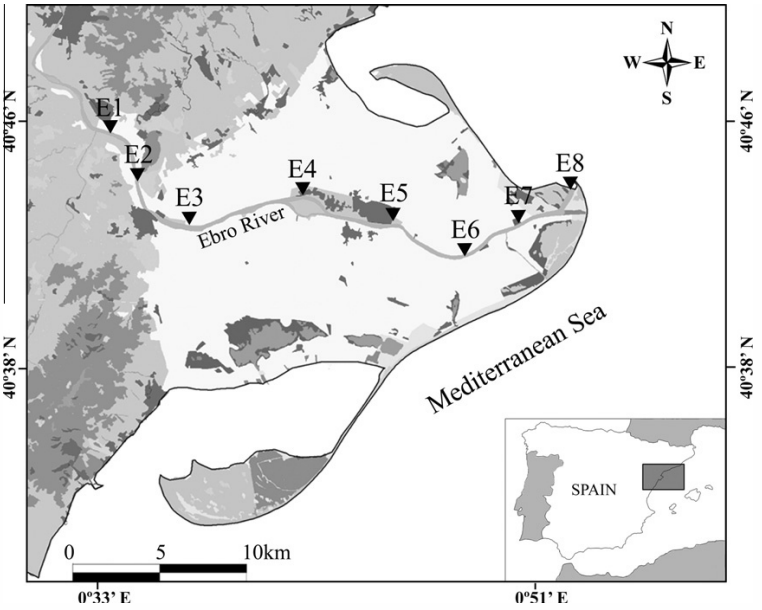

Fig. 1. Ebro Estuary map showing sampling sites.

nitrate ($N\text{-}NO_3^-$) was obtained from 1987 to 2008 from CHE database to show annual and monthly variability.

Total suspended solid concentration (TSS) and particulate organic matter (POM) were quantified according to UNE-EN 872 norm (AENOR, 1996). Phytoplankton chlorophyll *a* was extracted using 90% acetone and measured with a fluorimeter using Lorenzen formula (Lorenzen, 1966).

Table 1
Overview of diatom-based indices included in the present study. Code: indices abbreviation; source: publication from which the index was first described.

Code	Index	Source
CEE	Descy and Coste diatom index	Descy and Coste (1991)
DESCY	Descy index	Descy (1979)
DI-CH	Swiss diatom index	Buwal (2002)
EPI-D	Diatom-based eutrophication/ pollution index	Dell'Uomo (1996)
GENRE	Generic diatom index	Rumeau and Coste (1998)
IBD	Biological diatom index	Lenoir and Coste (1996)
IDAP	Artois-Picardie diatom index	Prygiel et al. (1996)
IDP	The Pampean diatom index	Gómez and Licursi (2001)
IPS	Specific pollution sensitivity index	Coste in Cemagref (1982)
L&M	Leclercq and Maquet index	Leclercq et al. (1987)
LOBO	LOBO index	Lobo et al. (2002)
SHE	Schiefele and Schreiner index	Schiefele and Schreiner (1991)
SID	Austrian saprobic index	Rott et al. (1997)
SLA	Sládeček index	Sládeček (1986)
TDI	Trophic diatom index	Kelly (1998)
TID	Austrian trophic index	Rott et al. (1999)
WHAT	Watanabe index	Watanabe et al. (1986)

2.4. Diatom identification

Benthic diatom samples were oxidised with H_2O_2 30% v/v a few hours in order to remove the organic matter and HCl^- 37% v/v was added to eliminate carbonates, as described in Renberg (1990). Clean valves were permanently mounted with Naphrax© (refractive index 1.74). Slides were examined using a LEICA DMI 3000B light microscope equipped with differential interference contrast (DIC) under oil immersion objective at ×100 magnification (n.a = 1.40). A minimum of 400 valves were counted at both natural and artificial replicates and identification of diatoms was done down to species level using specialised bibliography (e.g. Krammer and Lange-Bertalot, 1986, 1988, 1991a,b; Lange-Bertalot, 2001; Witkowski et al., 2000).

2.5. Data analysis

A principal component analysis (PCA) with all environmental data available (water depth, distance to the sea, water velocity, pH, DO_2, temperature, EC_{25}, water chlorophyll *a*, dissolved nutrients, TN, TP, TSS, POM and lower Ebro River flow records) was performed to reduce the dimensionality of the dataset and determine major environmental gradients in the Ebro Estuary. A total of 96 samples were included in the analysis using previously standardised variables (values minus its average and divided by its standard deviation). Varimax rotation was applied to maximise the variance of each of the factors, so the total amount of variance accounted for is redistributed over the extracted factors. Annual averages of daily flow and monthly fluctuation range of lower Ebro River flow were compared before and after reservoir construction by a one-way

Table 2
Diatom taxa with relative abundance (RA > 0.2%) and present in more than 5% of the Ebro Estuary samples.

Diatom taxa	% RA	Diatom taxa	% RA
Achnanthes amoena Hustedt	0.80	*Navicula* aff. *normaloides* Cholnoky	0.20
Achnanthidium minutissimum (Kützing) Czarnecki	1.42	*Navicula perminuta* Grunow	1.13
Achnanthes sp.	0.28	*Navicula* cf. *perminuta* Grunow	1.25
Amphora inariensis Krammer	0.29	*Navicula recens* (Lange-Bertalot) Lange-Bertalot	2.36
Amphora indistincta Levkov	2.22	*Navicula* aff. *recens* (Lange-Bertalot) Lange-Bertalot	0.27
Amphora aff. *luciae* Cholnoky	0.49	*Navicula reichardtiana* Lange-Bertalot	0.20
Amphora cf. *meridionalis* Levkov	0.61		
Amphora ovalis (Kützing) Kützing	0.21	*Navicula tripunctata* (O.F. Müller) Bory	0.36
Amphora pediculus (Kützing) Grunow	8.32	*Navicula veneta* Kützing	0.22
Amphora polita Krasske	0.58	*Nitzschia amphibia* Grunow	0.40
Amphora cf. *vetula* Levkov	1.83	*Nitzschia constricta* (Kützing) Ralfs	0.35
Bacillaria paxillifera (O.F. Müller) Hendey	2.57	*Nitzschia dissipata* (Kützing) Grunow	2.20
Cocconeis cf. *neothumensis* var. *marina* De Stefano, Marino & Mazzella	0.71	*Nitzschia filiformis* (W. Smith) Van Heurck	0.90
Cocconeis pediculus Ehrenberg	1.03	*Nitzschia* cf. *fonticola* (Grunow) Grunow	0.41
Cocconeis placentula var. *euglypta* (Ehrenberg) Grunow	14.62	*Nitzschia frustulum*[a] (Kützing) Grunow	8.19
Cocconeis placentula var. *placentula* Ehrenberg	1.34	*Nitzschia frustulum* var. *bulnheimiana* (Rabenhorst) Grunow	0.81
Cocconeis placentula var. *trilineata* (M. Peragallo & J. Héribaud) Cleve	5.30	*Nitzschia inconspicua* Grunow	5.44
Cyclotella meneghiniana Kützing	0.26	*Nitzschia microcephala* Grunow	0.21
Diploneis sp.	1.15	*Nitzschia palea* (Kützing) W. Smith	0.74
Eolimna subminuscula (Manguin) Moser, Lange-Bertalot & Metzeltin	0.43		
Fallacia clepsidroides Witkowski	0.41	*Nitzschia* cf. *palea* (Kützing) W. Smith	0.60
Gomphonema grovei var. *lingulatum* (Hustedt) Lange-Bertalot	0.40	*Nitzschia prolongata* Hustedt	0.34
Gomphonema cf. *minutum* (C. Agardh) C. Agardh	0.25	*Nitzschia* cf. *sociabilis* Hustedt	0.24
Gomphonemopsis obscura (Krasske) Lange-Bertalot	0.30	*Parlibellus* cf. *berkeleyi* (Kützing) Cox	0.23
Gomphonema parvulum (Kützing) Kützing	0.22	*Planothidium iberense* Rovira & Witkowski	0.43
Melosira varians C. Agardh	0.35	*Pleurosira laevis* (Ehrenberg) Compère	0.24
Navicula antonii Lange-Bertalot	1.21	*Psammothidium punctulatum* (Simonsen) Bukhtiyarova et Round	0.30
Navicula capitatoradiata Germain	0.21	*Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot	7.24
Navicula cryptotenella Lange-Bertalot	2.52	*Synedra ulna* (Nitzsch) Ehrenberg	0.21
Navicula cf. *cryptotenelloides* Lange-Bertalot	0.59	*Tabularia fasciculata* (C.Agardh) Williams & Round	2.67
Navicula gregaria Donkin	0.54	*Tabularia tabulata* (C.Agardh) Snoeijs	1.59
Navicula aff. *mollis* (W. Smith) Cleve	0.72	*Thalassiosira pseudonana* Hasle & Heimdal	0.22

[a] *Nitzschia frustulum* sensu lato: Our recent investigation (in preparation) suggest that specimens identified here as *N. frustulum* correspond to the initial cells of *N. inconspicua* after auxosporulation. However, we decided to maintain the division between the two taxa since nowadays they are still routinely identified as two different entities and, therefore, existing diatom indices also consider them separately.

ANOVA. PCA and one-way ANOVA were carried out using SPSS 19.0 for Windows.

Diatom assemblages were differentiated using hierarchical cluster analysis with Sorensen's similarity coefficient on relative abundance data and with a flexible beta ($\beta = -0.25$, Dufrêne and Legendre, 1997) as the linkage method. The statistical significance of between groups' differences was tested using the multi-response permutation procedure (MRPP). MRPP is a non-parametric procedure (does not require normality and homogeneity of variances) that tests the hypothesis of no differences in assemblage structure among groups. Sorensen coefficient was also used as the distance measure. Cluster analysis and MRPP procedure were run with PcOrd 5.0 for Windows. Diatom assemblages resulting from cluster analysis were visualised in a non-metric multidimensional scaling (NMDS) ordination space using PRIMER 6.0 for Windows.

Indicator species analysis (Dufrêne and Legendre, 1997) was used to identify diatom species which are indicators of the diatom assemblages obtained by cluster analysis and NMDS. Indicator species analysis combines the abundance and frequency of diatom species into an indicator value (IV) that varies from 0 to 100, with 100 being a perfect indicator value. IV is the maximum when all individuals are found in a single cluster (high specificity) and they are present in all the samples of the cluster (high fidelity) (Dufrêne and Legendre, 1997). IV were calculated using PcOrd 5.0 for Windows and only those IV statistically significant with Montecarlo permutations (999 permutations, $P < 0.05$) were included in the analyses.

EC_{25} optimum and tolerance for each indicator species were calculated with weighted averages using C2 software. Moreover, EC_{25} minimum, maximum and coefficient of variation (CV; [average/standard deviation] $\times$ 100) were also determined for each indicator species.

Seventeen diatom-based indices developed for river water quality evaluation were applied (Table 1). Most of these diatom indices are derived from the weighted average formula of Zelinka and Marvan (1961), which considers the sum of the species abundance influenced by their sensitivity and by their indicator value to different pollution gradients:

$$\mathrm{ID} = \frac{\sum \mathrm{Aj\ Ij\ Sj}}{\sum \mathrm{Aj\ Ij}}$$

where Aj = relative abundance of the species j; Ij = indicator value of the species j; and Sj = sensitivity value of the species j.

These indices were originally developed to assess nutrient and/or organic pollution in rivers and, recently, some of them have been adapted to fulfil the WFD requirements of assessing ecological status of freshwater ecosystems (Kelly et al., 2009).

Diatom indices were calculated using the OMNIDIA software version 4.1. Results from indices were assigned to five ecological status classes established by the WFD: values from 1 to 5 to "bad" status class; values from 5 to 9 to "poor" status class; values from 9 to 13 to "moderate" status class, from 13 to 16 to "good" status class and the "high" status class to values from 16 to 20.

Relationships between diatom indices and physicochemical variables were analysed with Spearman rank correlations using SPSS 19.0 for Windows. In all statistical analyses, except pH, environmental data were logarithmically transformed prior to analysis to reduce skewed distributions. Only species with a relative abundance (RA) > 0.2% and present in more than 5% of the samples were included in all analyses. Table 2 lists included species and their authorities.

3. Results

3.1. Hydrological and physicochemical variability of the lower Ebro River

Historical data of the lower Ebro River flow from early 1900s to 2008 is shown in Fig. 2. Since the construction of reservoirs (mainly since the construction of Mequinenza reservoir in 1964) and the expansion of the irrigated area, the lower Ebro River flow decreased 40% from an average of 565 m^3/s (1913–1963 period) to 356 m^3/s (1964–2008 period) (ANOVA, $P < 0.05$; Fig. 2a). A significant decrease in the monthly flow fluctuation range was also observed from an average of 892 m^3/s (1913–1963 period) to 487 m^3/s (1964–2008 period) after irrigation expansion and reservoirs construction (ANOVA, $P < 0.05$), and therefore the lower Ebro River flow is more stable now than before damming (Fig. 2b).

The lower Ebro River flow shows a seasonal pattern (Fig. 3a) both before and after reservoir construction, being higher and more variable from year to year in late winter and early spring, and decreasing in magnitude and variability in summer and early autumn. However, this seasonal pattern was slightly different in the present study (2007–2008) (Fig. 3b). The initial sampling campaign in 2007 was characterised by an increase in river flow in early spring, being at its maximum in April (monthly average of 982 m^3/s), corresponding to the opening of reservoir flood gates

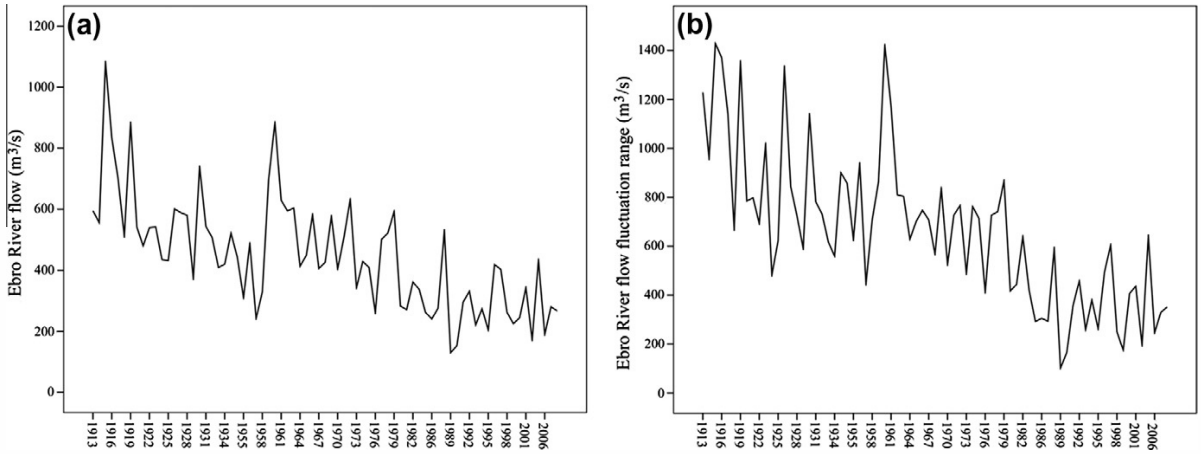

Fig. 2. (a) Lower Ebro River flow and (b) lower Ebro River flow fluctuation range (difference between maximum and minimum) from 1913 to 2008. The showed values are the annual averages for daily flow (a) and monthly fluctuation range (b). Data collected in Tortosa (40 km from Ebro River mouth) from Ebro Hydrographical Confederation (CHE).

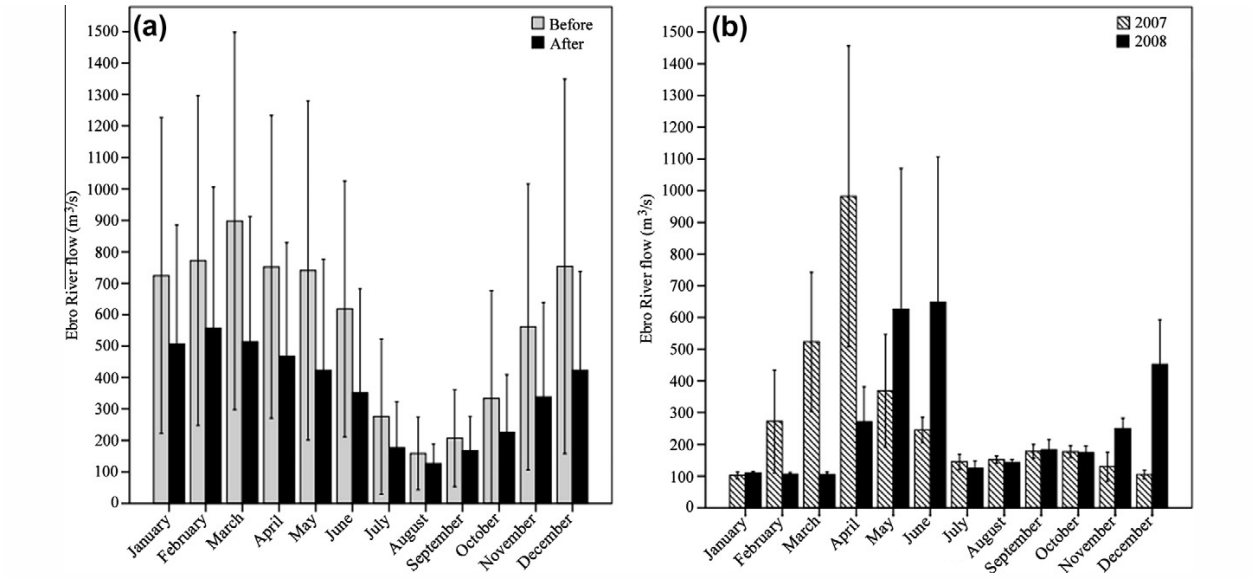

Fig. 3. (a) Monthly average and standard deviation of lower Ebro River flow from 1913 to 2008 before (grey bars) and after (black bars) construction of Mequinenza reservoir in 1964; (b) monthly average and standard deviation of lower Ebro River flow during the present study (2007 and 2008). Data collected in Tortosa (40 km from Ebro River mouth) from Ebro Hydrographical Confederation (CHE).

due to high water levels after a strong rainfall and melt-water period. Unfortunately, diatom sampling started in October 2007, and therefore there is no diatom data of this previous period. Lower Ebro River flow decreased that summer (monthly average <200 m^3/s) and did not increase in autumn and winter due to the drought suffered that year (water levels in Mequinenza reservoir were <50% from October 2007 to April 2008, according to CHE data). At the end of April 2008 and during May 2008 rainfalls filled reservoir levels again and reservoir flood gates were open from May until end of June 2008, with a monthly river flow average ~600 m^3/s.

Regarding dissolved nutrients, $P\text{-}PO_4^{3-}$ concentration in the lower Ebro River clearly decreased from average annual values around 0.2–0.3 mg/L $P\text{-}PO_4^{3-}$ in late 1980s to 0.05 mg/L $P\text{-}PO_4^{3-}$ in the present study (Fig. 4a); meanwhile $N\text{-}NO_3^-$ did not show any signs of decrease, being the historical average value of the $N\text{-}NO_3^-$ concentration of 2.43 mg/L (Fig. 4b).

3.2. Environmental gradients in the Ebro Estuary

The two first PCA factors accounted for the 37.83% of the total variation of the physicochemical and hydrological data considered (Table 3). The first PCA factor (21.51% of variation, Fig. 5) was well and negatively correlated with EC_{25} and water depth; and positively correlated with water velocity, DO_2, $N\text{-}NO_3^-$, TN and $Si\text{-}SiO_4^{4-}$. This factor separated deep water samples where the salt wedge was present from all the others, comprising both superficial and deep water non-salt-wedge samples. Salt-wedge water samples did not only have higher conductivities and lower nutrient concentrations, but also lower water velocities and lower DO_2. The decrease of DO_2 and water velocity was stronger near the salt-wedge tip, where the water can remain still for a long period and the oxygen consumption due to biological processes is higher. The second PCA factor (16.32% of variation, Fig. 5) was only related to hydrological variables, being strongly and positively correlated

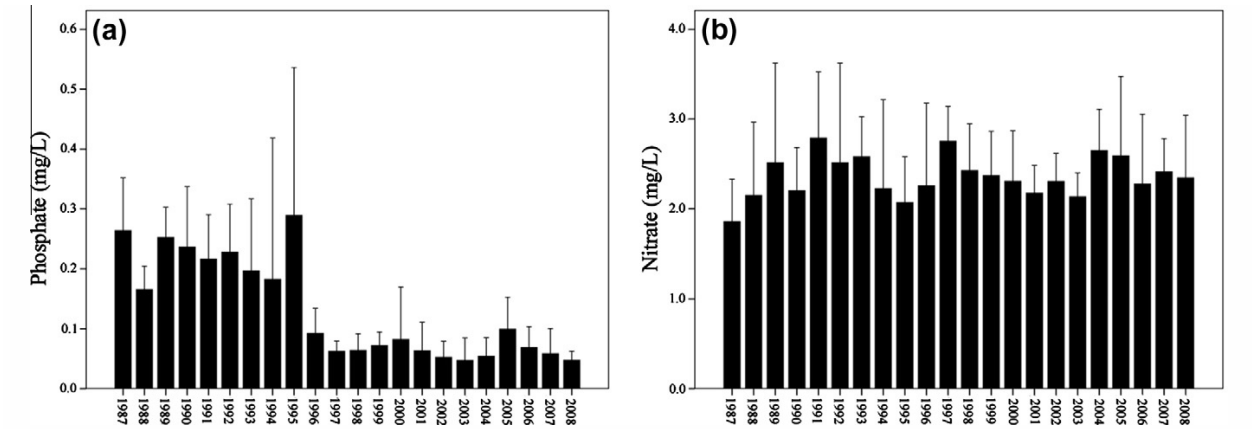

Fig. 4. (a) Historical dissolved phosphate ($P\text{-}PO_4^{3-}$) and (b) historical dissolved nitrate ($N\text{-}NO_3^-$) concentrations in the lower Ebro River from 1987 to 2008. Annual averages and standard deviation values are shown. Data collected in Tortosa (40 km from Ebro River mouth) from Ebro Hydrographical Confederation (CHE).

Table 3
Percentage of the explained variance and factor scores of the standardised environmental variables included in the PCA. Factor scores below 0.40 are not shown.

% Variance explained	PCA factor	
	1	2
	21.51	16.32
Depth (m)	−0.82	
DO_2 (mg/L)	0.53	
EC_{25} (mS/cm)	−0.84	
$N\text{-}NO_3^-$ (mg/L)	0.79	
TN (mg/L)	0.77	
$Si\text{-}SiO_4^{4-}$ (mg/L)	0.70	
Water velocity (m/s)	0.83	
Max. flow (m^3/s)		0.98
Flow fluctuation (m^3/s)		0.98
Average flow (m^3/s)		0.96

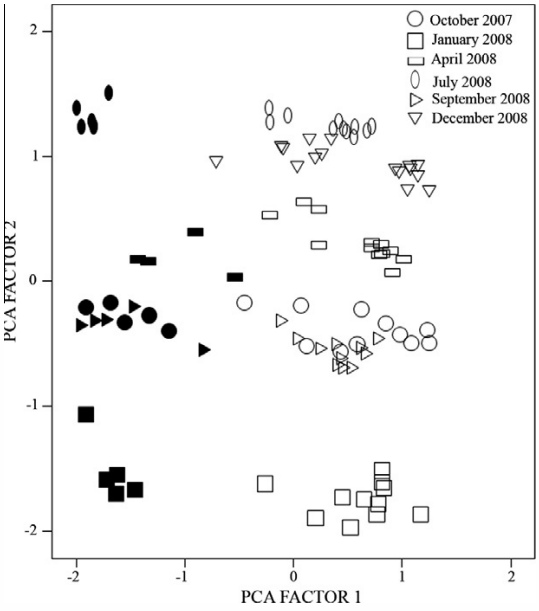

Fig. 5. Representations of the samples in the PCA factor space [1, 2]. Filled symbols represent deep samples with salt-wedge presence; empty symbols represent superficial samples and deep non-salt-wedge samples. Symbol shapes identify the sampling campaigns; circles: October 2007, squares: January 2008, rectangles: April 2008, ellipses: July 2008, right-sided triangles: September 2008, down-sided triangles: December 2008.

with the average, maximum and fluctuation range of lower Ebro River flow and which differentiated January samples from the rest. River flow was low (<200 m^3/s) and stable from summer 2007 to spring 2008 because of the scarce rainfall during that period. January 2008 samples had therefore been subjected to the longest period of low and stable flow until April, when rainfall returned. Thus, this axis could be related to stability of lower Ebro River flow during low flow periods, with the consequent stability of the salt wedge in its estuary.

3.3. Diatom assemblages and indicator species

A total of 160 diatom species were identified. From these, 62 species had a total relative abundance higher than 0.2%, occurring in more than 5% of samples and therefore were the species selected for the statistical analyses (Table 2). MRPP indicated significant differences between the identified groups in cluster analysis ($A = 0.199$, $P = 0.01$). Cluster analysis (view Supplementary material S1) and NMDS identified two main diatom assemblages (Fig. 6): one comprised of samples without or with weak marine influence (i.e. samples of riverine conditions, mostly upstream sites without salt-wedge intrusions); and the other comprised of samples with marine influence at deep water layers, due to salt-wedge presence, but also (although at a minor scale) at superficial layers due to the advective salt-wedge circulation and proximity to the sea (i.e. samples of estuarine conditions, mostly downstream sites).

Benthic diatom indicator species of both riverine and estuarine conditions were recognised through indicator species analysis (Supplementary material S2). From the 48 species that showed statistically significant indicator values (IV) after Montecarlo permutation test ($P < 0.05$), 17 species had high IV (>60%) and could therefore be considered as good indicator species of the two main ecological conditions identified in the Ebro Estuary. Thus, *Achnanthidium minutissimum* (IV = 77%), *Amphora pediculus* (IV = 80%), *Amphora* cf. *vetula* (IV = 78%), *Cocconeis placentula* var. *euglypta* (IV = 84%), *Cocconeis placentula* var. *trilineata* (IV = 85%), *Navicula antonii* (IV = 80%), *Navicula cryptotenella* (IV = 78%), *Navicula* cf. *cryptotenelloides* (IV = 63%) and *Nitzschia amphibia* (IV = 60%) emerged as good indicator species of riverine conditions in the Ebro Estuary. On the other hand, *Amphora polita* (IV = 69%), *Navicula gregaria* (IV = 61%), *Navicula* aff. *mollis* (IV = 69%), *Navicula perminuta* (IV = 60%), *Navicula recens* (IV = 61%), *Nitzschia constricta* (IV = 61%), *Nitzschia frustulum* (IV = 83%) and *Tabularia fasciculata* (IV = 65%) emerged as good indicator species for the estuarine conditions.

On the whole, the indicator species of riverine conditions tended to show higher frequency of occurrence within the condition (high fidelity) than those of estuarine conditions. The specificity of the 48 species considered for each of the two conditions was relatively high (>65%), though very high (>95%) specificity was only found in some indicator species of estuarine conditions such as *Achnantes amoena*, *Amphora* aff. *luciae*, *A. polita*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *Fallacia clepsidroides*, *Gomphonemopsis obscura*, *Navicula* cf. *perminuta*, *Nitzschia prolongata*, *Parlibellus* cf. *berkeleyi*, *Planothidium iberense* and *Psammothidium punctulatum*. Interestingly, most of these species showed a very low fidelity (they were only found in a reduced subset of samples within estuarine conditions), resulting in a low IV.

Optimum, tolerance, minimum, maximum and coefficient of variation for conductivity (EC_{25}) are also listed for all the indicator species of riverine and estuarine conditions in Supplementary material S2. Although most indicator species were present along the whole conductivity gradient, their EC_{25} optimum depended on the species considered. While indicator species of riverine conditions showed a more similar EC_{25} optimum (ca 2.45 mS/cm), indicator species of estuarine conditions exhibited a considerable variation in their EC_{25} optimum, ranging from 3.1 mS/cm in the case of *N. recens* up to 34.3 mS/cm for *Amphora* aff. *luciae*.

Interestingly, some indicator species of estuarine conditions such as *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *G. obscura*, *Parlibellus* cf. *berkeleyi* and *P. iberense* showed both the highest EC_{25} optimum values (>17 mS/cm) and the lowest coefficients of variation, and most of them also showed the highest specificity (>95%) and the lowest fidelity (<25%) values (i.e. they were present in particular samples within the condition).

3.4. Application of diatom-based indices

There are no specific indices for assessing ecological status of estuaries and other transitional waters using diatoms. Therefore, as a first step towards developing such an index, we evaluated

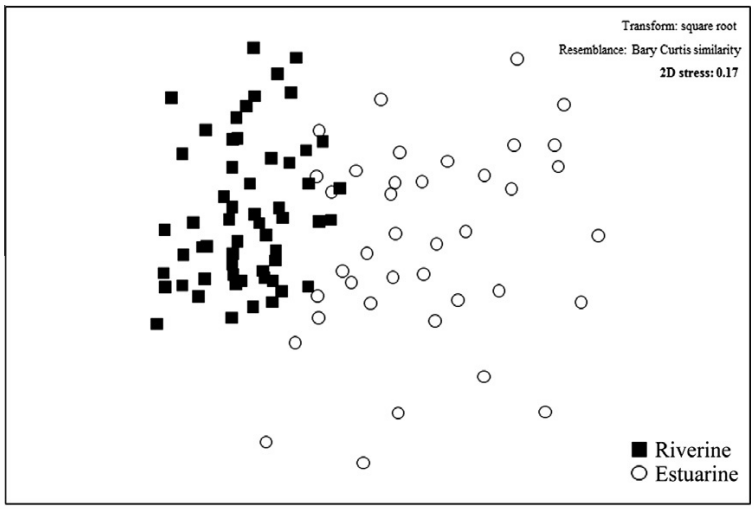

Fig. 6. Non-metric multidimensional scaling (NMDS) ordination plot of Ebro Estuary samples according to benthic diatom community composition. Filled squares: diatom assemblage of riverine conditions; empty circles: diatom assemblage of estuarine conditions.

Table 4
Ecological status class obtained for each sampling campaing after the application of diatom indices considered in OMNIDIA. Mod. = Moderate.

	CEE	DESCY	DI-CH	EPI-D	GENRE	IBD	IDAP	IDP	IPS	L&M	LOBO	SHE	SID	SLA	TDI	TID	WHAT
October 07	Mod.	Good	Poor	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Good	Mod.	Mod.	Mod.	Mod.	Poor	Poor	Good
January 08	Mod.	Good	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Bad	Poor	Good
April 08	Mod.	Good	Mod.	Mod.	Poor	Mod.	Mod.	Mod.	Good	Mod.	Mod.	Good	Mod.	Good	Bad	Poor	Good
July 08	Good	Good	Mod.	Mod.	Poor	Mod.	Mod.	Mod.	Good	Good	Good	Good	Good	Good	Poor	Poor	Good
September 08	Mod.	Good	Poor	Mod.	Mod.	Poor	Mod.	Mod.	Mod.	Good	Mod.	Mod.	Mod.	Mod.	Poor	Poor	Good
December 08	Good	High	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Mod.	Good	Mod.	Mod.	Mod.	Mod.	Poor	Poor	Good

the application of 17 diatom-based indices developed for rivers (see Section 2) to our sample set.

The ecological status classification of the Ebro Estuary depended entirely on which index was applied. For any given sampling campaign, different indices showed very different status class assessments of the Ebro Estuary (Table 4). Moreover, the status class resulting from the application of most indices also varied depending on the season considered. Extreme status classes were assigned to some sampling campaigns; January and April resulted in a "bad" class for TDI, meanwhile December was rated as "high" when applying DESCY. In general, trophic indices (TDI, TID) were the most severe ones, assigning "bad" or "poor" ecological status in all sampling campaigns (Table 4).

Diatom indices were also applied separately to the two groups of samples corresponding to riverine and estuarine conditions. In general, samples of estuarine conditions (most samples of downstream sites) showed lower ecological status values than samples of riverine conditions (mainly samples of upstream sites) (Supplementary material S3). For some indices (i.e. CEE, DESCY, EPI-D, GENRE, IBD, IDAP, IPS, L&M, TDI and WHAT) this differences resulted in an inferior ecological status class.

The percentage of indicator species considered in each index is represented in Table 5. On the whole, the indices applied included a higher percentage of indicator species of riverine conditions than of estuarine conditions. Except for IPS and TDI, the other 14 diatom indices that consider diatoms at species level did not take into account a very high percentage of indicator species of estuarine conditions (from 61% to up 96% indicator species were excluded depending on the index).

Spearman rank correlations were applied to all Ebro Estuary samples to find relationships between diatom-based indices and physicochemical variables (Table 6). Most indices were strongly and negatively correlated with conductivity ($P < 0.05$). The correlations of indices with nutrients were very weak and most of them showed an inverse response (positive correlation with nutrient concentration) to the expected one. Only IDP, L&M and trophic indices (TDI and TID) were negatively correlated with TP (IDP), $N\text{-}NO_2^-$ (L&M, TDI and TID) and $N\text{-}NO_3^-$ (TID), though all the correlation coefficient values were rather low (i.e. below 0.4).

In order to study the possible relationship between indices and nutrient enrichment without the strong effect of conductivity,

Table 5
Percentage (%) of indicator diatom species of riverine and estuarine conditions considered in each diatom-based index applied. Cons: indicator species considered; no cons: indicator species not considered. GENRE index was excluded since it only considers diatoms at genus level.

Index	Riverine conditions		Estuarine conditions	
	% Cons.	% No cons.	% Cons.	% No cons.
CEE	72.73	27.27	38.46	61.54
DESCY	59.09	40.91	11.54	88.46
DI-CH	72.73	27.27	26.92	73.08
EPI-D	72.73	27.27	30.77	69.23
IBD	81.82	18.18	34.62	65.38
IDAP	54.55	45.45	30.77	69.23
IDP	63.64	36.36	15.38	84.62
IPS	81.82	18.18	65.38	34.62
L&M	63.64	36.36	19.23	80.77
LOBO	50.00	50.00	7.69	92.31
SHE	72.73	27.27	30.77	69.23
SID	72.73	27.27	34.62	65.38
SLA	72.73	27.27	26.92	73.08
TDI	81.82	18.18	80.77	19.23
TID	77.27	22.73	34.62	65.38
WHAT	40.91	59.09	3.85	96.15
Average	68.20	31.80	30.80	69.20

Table 6
Spearman rank correlations between diatom indices and water physicochemical parameters in the Ebro Estuary. Only significant correlations at $P < 0.05$ are shown. Negative correlations of indices and nutrients are highlighted in bold. Vel. water velocity.

	pH	Temp (°C)	DO_2 (mg/L)	EC_{25} (mS/cm)	TP (μg/L)	$N\text{-}NO_2^-$ (μg/L)	$N\text{-}NO_3^-$ (mg/L)	TN (mg/L)	Chl *a* (μg/L)	TSS (mg/L)	POM (%)	Vel. (m/s)
CEE				-0.53								
DESCY	−0.24			−0.34			0.30	0.26	−0.23	0.28	−0.27	
DI-CH	−0.52	−0.36		−0.20		0.31	0.37	0.30	−0.31	0.29		
EPI-D				−0.56			0.23					
GENRE				−0.34								
IBD				−0.56								
IDAP				−0.62			0.29	0.22				
IDP	−0.57	−0.36	0.22		**−0.33**	0.48	0.44	0.29		0.34	−0.21	0.25
IPS				−0.59			0.31	0.25				
L&M				−0.54	0.29	**−0.25**						−0.21
LOBO		0.24							0.23			
SHE	−0.22			−0.36			0.29	0.22				0.32
SID				−0.37			0.23			0.26		
SLA			−0.23	−0.20					−0.24			
TDI	0.32		−0.21		0.23	**−0.30**	**−0.24**					−0.29
TID	0.23			−0.54		**−0.28**						
WHAT				−0.56			0.30	0.25				

Table 7
Spearman rank correlations between diatom indices and physicochemical parameters in upstream superficial samples. Only significant correlations ($P < 0.05$) are shown. Significant negative correlations between diatom indices and nutrients are highlighted in bold. Vel. water velocity.

	pH	Temp (°C)	DO_2 (mg/L)	EC_{25} (mS/cm)	TP (μg/L)	$N\text{-}NH_4^+$ (μg/L)	$N\text{-}NO_2^-$ (μg/L)	$N\text{-}NO_3^-$ (mg/L)	TN (mg/L)	Chl *a* (μg/L)	TSS (mg/L)	POM (%)	Velo (m/s)
CEE				−0.45									
DESCY	−0.33	−0.39		−0.33				0.39	0.40		0.28	−0.34	
DI-CH	−0.53	−0.40		−0.36				0.39	0.35	−0.36			
EPI-D				−0.53									
GENRE				−0.34						−0.34			
IBD				−0.54									
IDAP	−0.51			−0.53				0.41	0.34	−0.30			
IDP	−0.59	−0.32			**−0.33**		0.44	0.48	0.30		0.30		
IPS	−0.48			−0.55				0.44	0.35	−0.33			
L&M				−0.49	0.29	**−0.30**	**−0.33**						
LOBO			−0.35			0.30							
SHE	−0.38			−0.48				0.41	0.37	−0.29	0.31		0.39
SID				−0.46						−0.32			
SLA				−0.36									
TDI	0.50	0.37					**−0.51**	**−0.49**	**−0.33**				
TID				−0.48			**−0.39**						
WHAT	−0.44			−0.35				0.35	0.30	−0.33			

Table 8
Spearman rank correlations between diatom indices and physicochemical variables at salt-wedge deep samples. Only significant correlations at $P < 0.05$ are shown. Significant negative correlations between diatom indices and nutrients are highlighted in bold.

	pH	Temp (°C)	EC_{25} (mS/cm)	TP (μg/L)	$N\text{-}NH_4^+$ (μg/L)	$N\text{-}NO_2^-$ (μg/L)	$N\text{-}NO_3^-$ (mg/L)	TN (mg/L)	$Si\text{-}SiO_4^{4-}$ (mg/L)	Chl *a* (μg/L)	TSS (mg/L)
CEE										0.66	
DI-CH	−0.72	−0.71	−0.79			0.65					
EPI-D		0.62	0.73				**−0.66**			0.65	
IBD			0.75				**−0.71**				−0.82
IDAP						**−0.72**		**−0.62**	**−0.62**		
IDP	−0.68		−0.65								
IPS			0.73			**−0.65**					
L&M		0.75	0.71	0.66							
SHE					**−0.84**				**−0.62**		
SID					**−0.64**				**−0.61**	0.74	
TDI								**−0.66**			
TID			0.69			**−0.70**	**−0.72**		**−0.61**		
WHAT			0.70			**−0.62**	**−0.71**			0.67	

Spearman rank correlations were also applied in two groups of samples with more or less stable conductivity: (i) upstream superficial samples without salt-wedge influence (Table 7); and (ii) deep water samples with salt-wedge presence (Table 8). The negative correlation between indices and conductivity was still very strong in the case of upstream superficial sites, and once more only very few indices showed negative response to nutrients (i.e. IDP, L&M, TDI and TID). Correlation between conductivity and indices in salt-wedge samples did not show a clear pattern, being positively or negatively correlated depending on the index considered (Table 8). Negative correlations between diatom-based indices and nutrient concentrations increased when salt-wedge samples

were considered alone, as it was the case of EPI-D, IBD, IDAP, IPS, SHE, SID, TDI, TID and WHAT.

4. Discussion

4.1. Identification of potential human pressures in the Ebro Estuary

Results indicate that, at present, the main anthropogenic pressure in the Ebro Estuary is associated with the hydrological alteration of the lower Ebro River (i.e. increased salt-wedge presence and river flow stability). Both salt-wedge presence and periods of low and stable flows are natural processes occurring in a stratified estuary with scarce and seasonal rainfall periods. However, increased irrigation and reservoir construction in early 1960s caused a decrease of 40% of the lower Ebro River flow (Ibáñez et al., 1996; Sabater et al., 2008; Sierra et al., 2004) and therefore flow is lower and more stable now than before intensive water use. Flow regulation increased the presence of the salt wedge during most part of the year and reduced changes in its position (Ibáñez et al., 1996, 1997), causing a potential impact on biological communities, not only at a spatial scale, because the salt wedge is found further upstream than before reservoir construction, but also at a temporal scale, because the salt wedge is sometimes present during meltwater and rainfall periods.

Regarding nutrient concentrations, the lower Ebro River and its estuary showed severe eutrophication due to phosphorus enrichment during the 1980s and 1990s. This situation changed since 1995–1996, when phosphorus concentration suddenly decreased from values of 0.2–0.3 mg/L P-PO_4^{3-} to values of 0.05 mg/L P-PO_4^{3-} in the present study. This decrease in phosphorus could be explained by the construction in mid 1990s of waste water treatment facilities in the main cities of the middle Ebro basin together with the banning of detergents with phosphates; these may have reduced eutrophic conditions and therefore decreased phytoplankton concentrations (Ibáñez et al., 2008, 2012; Torrecilla et al., 2005). However, the same trend has not been observed for nitrate concentration, likely due to its origin from non-point sources from agriculture, which are much more difficult to control (Torrecilla et al., 2005). Nowadays, nutrient concentrations in the lower Ebro River and more specifically in its estuary (i.e. the last 40 km) are relatively low and show low seasonal variability when compared to other large Mediterranean rivers (Blanco et al., 2008; Leira and Sabater, 2005; Tornés et al., 2007). Therefore, it seems that at present, nutrient enrichment may not constitute the main pressure for diatom communities in the Ebro Estuary.

4.2. Diatom assemblages and indicator species in the Ebro Estuary

The benthic diatom community responds to the main environmental gradients and human pressures in the Ebro Estuary, namely the marine influence as a result of the river flow dynamics, which is the main factor driving species composition and distribution (Rovira et al., 2012). Conductivity thresholds between indicator species of riverine and estuarine conditions in Ebro Estuary are around 3 mS/cm which is in agreement with previous studies recording a change in diatom community composition around salinities of 3 ppt (Hammer, 1986; Ryves et al., 2002). Interestingly, though different substrata was used (artificial and natural), benthic diatom community in the Ebro Estuary is more affected by the marine influence than by substrata type (Rovira et al., 2012).

A few decades ago, under eutrophic conditions, nutrient enrichment probably influenced diatom community much more than now, as it has been found in other estuarine systems (Gómez et al., 2009; Underwood et al., 1998). However, since no benthic diatom data exists from these eutrophic conditions, either of the lower Ebro River or its estuary, it is very difficult to hypothesise how the diatom community responded to nutrient concentration in the past and, more importantly, to have a comparative approach in which the nutrient gradient is wide enough to show an impact on the diatom community composition and distribution.

In general, the specificity of indicator diatom species for each environmental condition was fairly high (>70% in most cases), showing that it is possible to identify and rely on these species as indicators of each type of condition. *Amphora minutissimum*, *Amphora pediculus*, *Amphora* cf. *vetula*, *Cocconeis placentula* var. *euglypta*, *Cocconeis placentula* var. *trilineata*, *Navicula antonii*, *Navicula cryptotenella*, *Navicula* cf. *cryptotenelloides* and *Nitzschia amphibia* indicate riverine conditions, these are situations with no (or with very weak) marine influence. These riverine conditions are mainly found in upstream sites of the Ebro Estuary. On the other hand, *Amphora polita*, *Navicula gregaria*, *Navicula* aff. *mollis*, *Navicula perminuta*, *Navicula recens*, *Nitzschia constricta*, *Nitzschia frustulum* and *Tabularia fasciculata* indicate estuarine conditions in the Ebro Estuary (i.e. situations with marine influence at both superficial and deep water layers).

However, only few species reached specificities of 100%, revealing that, even though indicator species showed a strong preference for one of the two main environmental conditions in Ebro Estuary (i.e. riverine and estuarine), most of them can also be found along the whole estuary, even when the other environmental condition is prevailing. Therefore, and as it can also be quickly withdrawn from the species EC_{25} minimum, maximum and coefficient of variation, the indicator species of Ebro Estuary are tolerant to a wide range of salinity conditions, as it has been found in other similar fluctuating systems (Bate and Smailes, 2008; Sullivan and Currin, 2000; Trobajo et al., 2004; Underwood, 1994).

Due to the interaction between river flow and the sea and the consequent salt-wedge dynamics, estuarine conditions are less homogenous (both at spatial and temporal scales) than riverine conditions, and therefore, the distribution of some indicator species is restricted to very particular situations. This is the case of *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *Gomphonemopsis obscura*, *Parlibellus* cf. *berkeleyi* and *Planothidium iberense*. These species are only found in situations of well-established salt wedge and consequently showing very high specificity (the salt wedge is only present in estuarine conditions) but very low fidelity (well-established salt wedge is not always present in estuarine conditions; and when present do not affect all samples). Because these diatom species are not only abundant in deep samples but also in their corresponding superficial samples, they can be considered indicative at both water layers of a long term salt-wedge presence. Thus, our results show that not only species having high indicator value (i.e. showing high specificity and high fidelity values) can be considered good indicators of environmental conditions but that species showing other combination of fidelity and specificity may be also useful indicators of a particular situation within a given condition. The same conclusion has also been previously reached for diatom species of Mediterranean streams (Tornés et al., 2007).

The categorisation of indicator diatom species of estuarine conditions allows the detection of hydrological pressures caused by flow regulation and abstraction and the consequent alteration of salt-wedge dynamics in a highly stratified estuary. However, because the occurrence of most indicator species is not strictly restricted to a particular condition, it would be much more reliable to consider the relative abundance of the indicator species all together rather than considering the abundance of each indicator species separately. Thus, in the Ebro Estuary two possible diatom species combinations would allow the detection of hydrological pressures both at spatial and temporal scales: (i) high abundances of *A. polita*, *N. gregaria*, *Navicula* aff. *mollis*, *N. perminuta*, *N. recens*,

N. constricta, *N. frustulum* and *T. fasciculata* (typical from estuarine conditions) when found in upstream sites (or in downstream sites during periods of expected high flow) would indicate a considerable flow reduction affecting diatom communities; and (ii) high abundances of *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *G. obscura*, *Parlibellus* cf. *berkeleyi* and *P. iberense* in downstream sites would indicate a well-established and long-lasting salt wedge. This situation occurs naturally in summer, but can also be present in other seasons where human alterations in hydrological conditions have prevented the salt wedge from being flushed away. An unsolved issue is to be able to discriminate the response of diatom assemblages to anthropogenic (i.e. flow regulation and abstraction) versus natural stresses (i.e. drought conditions), as well as the characterisation of reference conditions under a more fluctuating dynamics of the salt wedge.

4.3. Diatom indices evaluation

None of the studied indices were strongly and negatively correlated with P-PO_4^{-3} and only the trophic indices (i.e. TDI and TID) showed significant negative correlation with the dissolved nitrogen (i.e. N-NO_3^- and N-NO_2^-). Besides, TID accounted for the highest negative correlations with NO_3^- and NO_2^- when only salt-wedge samples were considered (N is usually the limiting nutrient in salt water). However, trophic indices were originally developed to detect phosphorus enrichment instead of nitrogen (Kelly, 1998; Rott et al., 1999). Moreover, the obtained low class values ("poor" and "bad" classifications) do not correspond to the trophic status of the lower Ebro River when phosphorus concentrations are considered, which would be classified as mesotrophic according to Dodds et al. (1998) classification of temperate rivers. Nowadays, the narrow range of nutrient variability in the lower Ebro River and particularly in the Ebro Estuary could be one of the factors explaining the weak relationship found between nutrients and diatom indices, resulting in unrealistic water quality assessments of the Ebro Estuary.

Other important limitations were also identified when the existing diatom indices were applied. Because most of indices have been developed from and for North-Central European freshwater systems, several ecologically important species of the Ebro Estuary are not included in most of the indices (e.g. *Amphora* aff. *luciae*, *A. polita*, *G. obscura* or *Navicula* aff. *mollis*, all considered to be brackish or marine species; Archibald, 1983; Hartley, 1986; Witkowski, 2000). Furthermore, some of the species considered in the indices may not indicate the same environmental situation in freshwater systems as in estuaries. For example, in freshwater systems *N. frustulum* has been found to be very abundant in situations with high levels of organic matter (Leira and Sabater, 2005), high inorganic nutrient concentrations (Van Dam et al., 1994), and high conductivities resulting from human activities (Rimet, 2009; Van Dam et al., 1994). However, as estuaries are characterised by much greater fluctuating conditions than rivers and by an intrinsic level of nutrients and organic matter (Trobajo and Sullivan, 2010), the high abundance of *N. frustulum* and accompanying taxa (*A. polita*, *N. gregaria*, *Navicula* aff. *mollis*, *N. perminuta*, *N. recens*, *N. constricta* and *T. fasciculata*) are indicative of estuarine conditions without having to imply a decrease in the ecosystem health or ecological status.

In conclusion, our results show that none of the diatom indices developed for river quality evaluation and later adapted to assess the ecological status of these ecosystems (Kelly et al., 2009) can properly assess the ecological status of the Ebro Estuary. Thus, the application of freshwater indices in estuaries and other transitional waters should be done very cautiously. The only ones that show the expected response to nutrients and consider a high percentage of indicator species are the trophic indices (TDI and TID), which could be potentially adapted to estuarine ecosystems after some adjustments in their species indicator values. Before the application of any diatom index some relevant issues need to be clarified, such as the characterisation of reference conditions, the distinct (or similar) effects of natural and anthropogenic stressors on diatom communities, and the type of response of diatom assemblages to an increased hydrological stability due to anthropogenic stressors (i.e. flow regulation). Therefore, in order to implement a diatom biomonitoring system for transitional waters, further research is needed to understand the role of diatom communities in this type of ecosystems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.marpolbul.2012.01.005.

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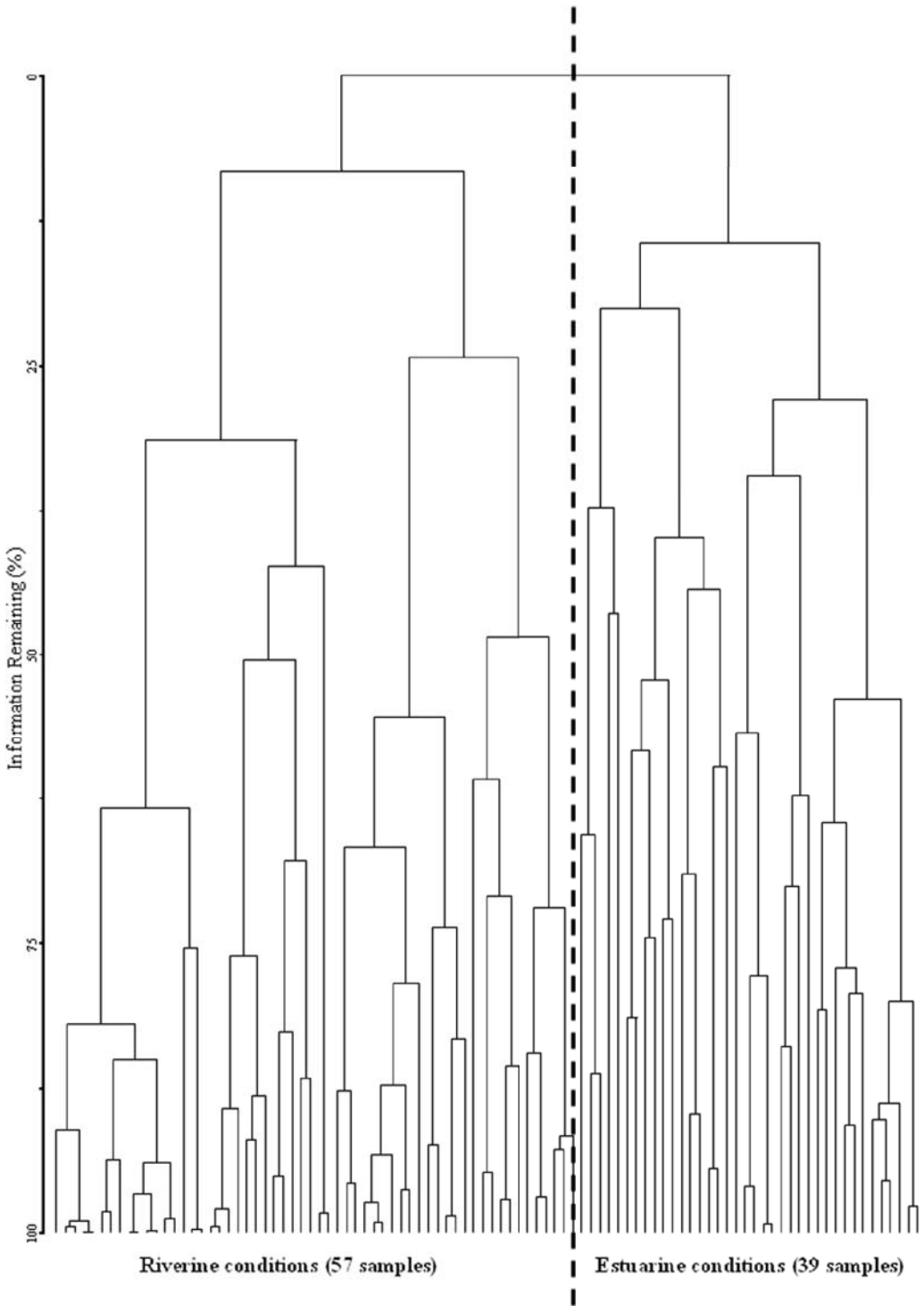
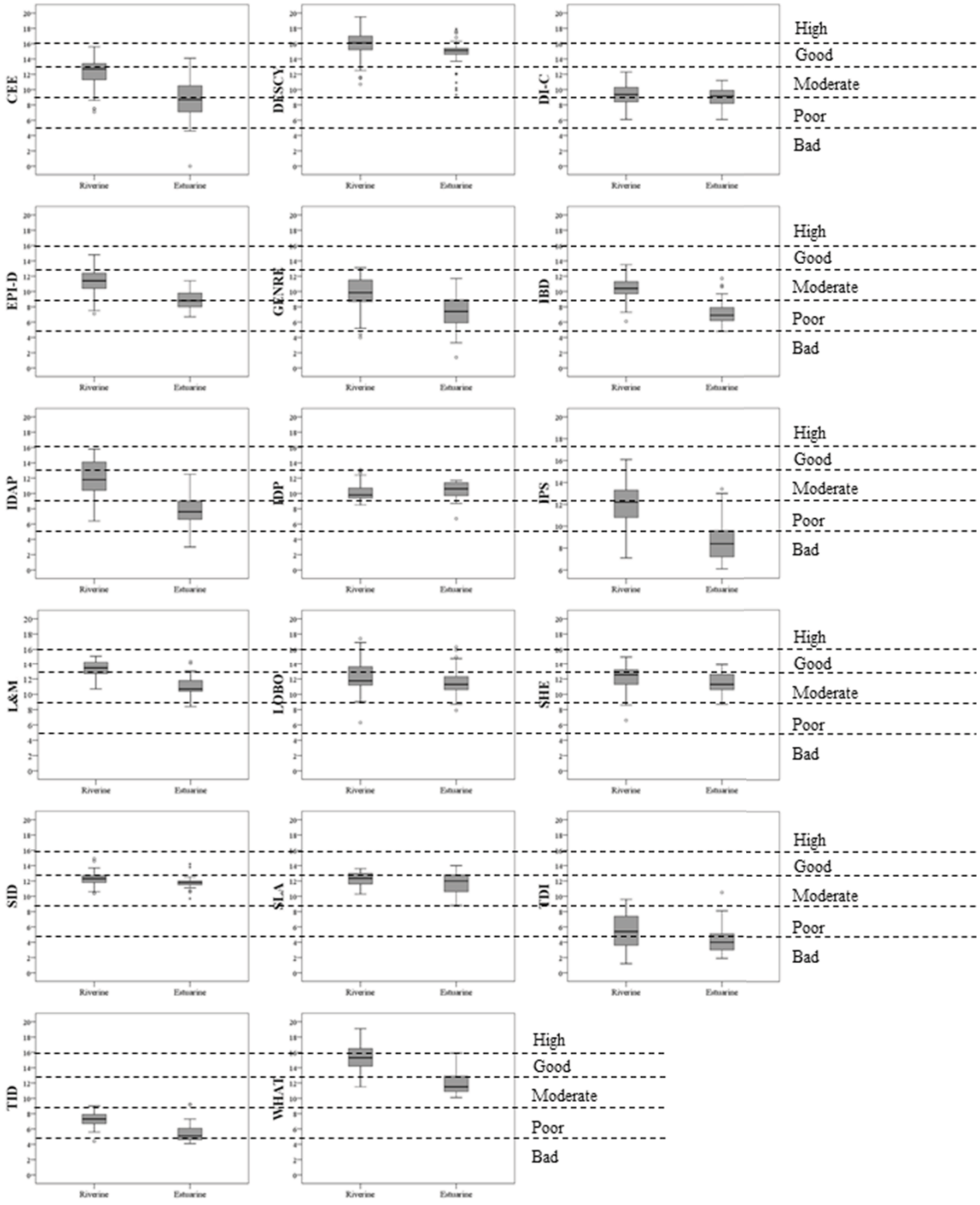
				EC_{25} (mS/cm)				
Riverines pecies	**IV**	**S**	**F**	**Op.**	**Tol.**	**Min.**	**Max.**	**CV**
Cocconeis placentula var. *trilineata*	85	88	97	2.5	1.6	0.8	52.6	243
Cocconeis placentula var. *euglypta*	84	84	100	2.5	1.9	0.8	52.6	210
Amphora pediculus	80	80	100	2.5	2.0	0.8	52.6	216
Navicula antonii	80	84	95	2.4	1.7	0.8	52.6	213
Navicula cryptotenella	78	80	98	2.3	1.6	0.8	52.6	238
Amphora cf. *vetula*	78	89	88	2.6	1.8	0.8	52.6	240
Achnanthidium minutissimum	77	91	84	2.4	1.8	0.8	52.6	246
Navicula cf. *cryptotenelloides*	63	83	76	2.3	1.6	0.8	51.6	281
Nitzschia amphibia	60	79	76	2.9	2.4	0.8	52.6	252
Cocconeis pediculus	59	67	88	2.7	1.7	0.8	52.6	229
Cocconeis placentula var. *placentula*	56	93	60	2.2	1.6	0.8	52.6	210
Amphora indistincta	54	89	60	2.4	2.0	0.8	52.6	258
Amphora cf. *meridionalis*	53	79	67	2.9	2.6	0.8	52.6	214
Navicula capitatoradiata	50	93	53	2.1	1.3	0.8	42.9	301
Amphora ovalis	49	84	59	2.7	2.2	0.8	52.6	253
Navicula tripunctata	47	67	71	2.4	1.6	0.8	51.9	257
Nitzschia cf. *fonticola*	45	65	69	2.8	2.2	0.8	43.1	256
Gomphonema parvulum	38	79	48	2.4	1.8	0.8	52.6	264
Navicula reichardtiana	37	83	45	2.2	1.5	0.8	51.9	275
Melosira varians	36	84	43	2.2	1.4	0.8	51.9	279
Synedra ulna	34	82	41	2.4	1.6	0.8	51.9	234
Gomphonema cf.*minutum*	30	79	38	2.2	1.6	0.8	52.6	309
				EC_{25} (mS/cm)				
Estuarine species	**IV**	**S**	**F**	**Op.**	**Tol.**	**Min.**	**Max.**	**CV**
Nitzschia frustulum	83	90	92	6.0	3.4	0.8	52.6	213
Amphora polita	69	97	71	4.9	3.4	1.0	51.9	158
Navicula aff. *mollis*	69	82	84	4.5	2.6	1.0	51.9	176
Tabularia fasciculata	65	73	89	3.7	2.4	0.8	42.9	200
Navicula recens	61	65	95	3.1	2.0	0.8	52.2	219
Navicula gregaria	61	89	68	11.2	4.0	0.8	52.2	166
Nitzschia constricta	61	93	66	6.7	3.2	1.0	52.2	142
Navicula perminuta	60	95	63	6.4	2.9	0.8	51.9	156
Nitzschia cf. *palea*	56	88	63	4.3	2.5	0.8	52.2	233
Diploneis sp.	56	96	58	28.0	4.0	1.0	52.2	144
Achnanthes sp.	52	93	55	8.2	2.7	0.8	51.9	142
Tabularia tabulata	48	76	63	3.4	1.6	0.8	42.9	200
Achnanthes amoena	46	98	47	8.3	5.2	0.8	52.6	133
Nitzschia prolongata	45	96	47	7.1	4.9	1.0	52.2	154
Navicula veneta	37	79	47	3.2	2.2	0.8	52.6	209
Navicula aff. *recens*	37	87	42	3.4	1.7	1.0	51.9	231
Amphora aff. *luciae*	34	99	34	34.3	3.4	1.0	52.2	95
Gomphonema grovei var. *lingulatum*	32	87	37	2.8	2.3	0.8	51.9	200
Navicula cf. *perminuta*	31	97	32	4.8	2.7	1.0	51.6	192
Fallacia clepsidroides	26	99	26	9.5	3.5	1.0	51.6	105
Gomphonemopsis obscura	23	96	24	26.4	3.2	0.8	42.9	123
Psammothidium punctulatum	18	100	18	8.2	2.7	1.0	39.9	109
Cocconeis cf. *neothumensis* var. *marina*	18	100	18	17.2	3.3	4.5	39.9	74
Parlibellus cf. *berkeleyi*	18	100	18	23.7	5.0	1.0	51.6	87
Navicula aff. *normaloides*	17	91	18	17.4	5.5	1.0	45.3	153
Planothidium iberense	16	100	16	17.2	3.4	3.5	39.9	109

Supplementary Material S2. Indicator values (IV) and conductivity (EC_{25}) optimum, tolerance, minimum, maximum and coefficient of variation (CV) of indicator diatom species of riverine and estuarine conditions in the Ebro Estuary. Only species with statistically significant IV ($P < 0.05$) are considered. Fidelity (F) and Specificity (S) values are also shown.



Supplementary Figure 1. Cluster dendogram of the Ebro Estuary samples according to similarity in diatom composition



Supplementary Figure 2. Box-plot diagram of diatom-based indices applied separately in samples from riverine and estuarine conditions of the Ebro Estuary. Dashed lines represent the thresholds between ecological status classes.

Chapter 3

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Effects of salinity on growth and on valve morphology of five estuarine diatoms

Rosa Trobajo,[1]* **Laia Rovira,**[1] **David G. Mann**[2] **and Eileen J. Cox**[3]

[1]Aquatic Ecosystems, Institute for Food and Agricultural Research and Technology (IRTA), Crta de Poble Nou Km 5.5, Sant Carles de la Ràpita, Catalunya, Spain, [2]Royal Botanic Garden Edinburgh, Edinburgh EH3 5LR, Scotland, and [3]Department of Botany, Natural History Museum, London SW7 5BD, UK

SUMMARY

The effects of salinity on the growth and valve morphology of five benthic estuarine diatoms (*Nitzschia pusilla*, *N. frustulum*, *N. palea*, *N. filiformis* var. *conferta* and *Eolimna subminuscula*), isolated from both freshwater and brackish/marine habitats, were investigated. The four *Nitzschia* strains grew well over a broad salinity range, though some (*N. pusilla, N. frustulum*) showed a broader salinity range tolerance (from fully saline down to at least 9.5 ppt) than others (*N. palea, N. filiformis* var. *conferta* had reduced growth at salinities of 16 ppt and above). Salinity significantly affected the valve morphology of the five strains studied. However, there was no consistent pattern in either the morphological characters affected or the direction of the effects. Although significant, the effects of salinity on valve morphology were very small and therefore it seems that the taxonomic usefulness of some of the classical taxonomical characters is not undermined.

Key words: culture, diatoms, estuarine, morphological variability, *Nitzschia*, phenotypic plasticity, salinity.

INTRODUCTION

Diatoms have proved to be excellent environmental indicators, since they react with speed and sensitivity to changes in aquatic ecosystems (for detailed reviews on the subject, see Smol & Stoermer 2010), and due to the fact that their cell walls are preserved in sediments for many years, they have also become useful for studying and interpreting past conditions. In particular, diatoms are the most widely used indicators for palaeosalinity reconstructions (Fritz 1990; Fritz *et al*. 1991; Juggins 1992; Cumming & Smol 1993; Ryves *et al*. 2004; Saunders 2010).

Salinity has often been considered to be an important factor determining diatom distribution in estuaries, and in fact, tolerance to changes in salinity (caused, for example, by tides, changing freshwater inputs and rainfall) has been inferred, from field studies, to be a prerequisite for most diatoms living in estuaries and coastal wetlands (Round 1960; Underwood 1994; Sullivan & Currin 2000; Trobajo *et al*. 2004a). However, culture-based studies on the growth of these species under wide salinity regimes are required to better interpret the role of the salinity in determining their natural distributions.

Salinity can also affect morphology, and several experimental works (Geissler 1970a,b, 1982, 1986; Schultz 1971; Schmid 1976; Jahn 1986; Wendker & Geissler 1988; Cox 1995; Trobajo *et al*. 2004b) have shown how changes in salinity can modify the valve morphology of a number of diatoms. These results are of particular importance in relation to estuarine diatoms, since many of the common taxa inhabiting these systems are small and taxonomically very difficult species, particularly of *Nitzschia* and *Navicula* spp. (Trobajo 2007). Data on the phenotypic plasticity of these species are essential to improve their taxonomy, and sound taxonomy is essential for any ecological and applied work on estuarine and coastal diatoms.

The aims of this study are to determine the effects of salinity on the morphology of five species of estuarine diatoms (*Nitzschia pusilla* Grunow, *N. frustulum* (Kützing) Grunow, *N. palea* (Kützing) W. Smith, *N. filiformis* (W. Smith) Van Heurck var. *conferta* (Richter) Lange-Bertalot and *Eolimna subminuscula* (Manguin) Moser) and to monitor their growth under different salinity regimes.

MATERIALS AND METHODS

In 2009, samples of microphytobenthos were collected from brackish and freshwater habitats of the Ebro Delta and Lower Ebro River. Diatom cultures were established by micropipetting single cells into Petri dishes containing f/2 medium (McLachlan 1973) or Woods Hole MBL medium (Nichols 1973), depending on the original

*To whom correspondence should be addressed.
Email: rosa.trobajo@irta.cat
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salinity of the samples from which the clones were isolated. Table 1 gives the origin and isolation details of the clones. Monocultures of *Nitzschia pusilla*, *N. frustulum*, *N. palea*, *N. filiformis* var. *conferta* and *Eolimna subminuscula* were established by further subculturing. Species were identified using Krammer and Lange-Bertalot (1986, 1988; *E. subminuscula* as *Navicula subminuscula* Manguin). The five species studied here are common in the benthic community of the Ebro Estuary (Rovira *et al*. 2009) and they have also been reported as common in other estuaries and coastal wetlands and lagoons (Drum & Webber 1966; Sullivan 1982; Bak *et al*. 2001; Trobajo 2007). However, and in accordance with the original growth medium of each clone (Table 1), *N. pusilla* and *N. frustulum* are referred to in this study as 'brackish' species, while *N. palea*, *N. filiformis* var. *conferta* and *E. subminuscula* are referred to as 'freshwater' ones.

A range of salinities (see Tables 2,3) were obtained by mixing appropriate proportions of MBL and f/2 media. f/2 medium was prepared using filtered natural sea water (32 ppt) from the North Sea. Cells of each

Table 1. Origin and isolation information for the five clones used in this study

Taxon	Original strain designation	Origin Habitat	Sample from	Salinity (ppt)	Isolation date	Original growth medium
Nitzschia pusilla	IRTA01	La Trinitat salt works pond (Ebro Delta)	Algal mat	37.32	Feb-10	f/2
Nitzschia frustulum	IRTA11	IRTA aquaculture wastewater lagoon (Ebro Delta)	Stone	31.26	Nov-09	f/2
Nitzchia palea	IRTA16	Lower Ebro River (by the Ginestar island)	*Potamogeton pectinatus*	0.33	Sep-09	MBL
Nitzschia filiformis var. *conferta*	IRTA20	Ebro Estuary (Ebro Delta)	Surface of tree trunk	1.89	Apr-09	MBL
Eolimna subminuscula	IRTA22	Lower Ebro River (by the Ginestar island)	Stone	0.38	Oct-09	MBL

The original strain designations correspond to voucher slides and unmounted material held in the Royal Botanic Garden Edinburgh.

Table 2. Growth of the two 'brackish' clones after 21 days under different salinity regimes, assessed visually

Salinity (ppt)	*Nitzschia pusilla*		*Nitzschia frustulum*	
	R1	R2	R1	R2
32	+++	+++	+++	+++
27	+++	+++	+++	+++
22	+++	+++	+++	+++
16	+++	+++	+++	+++
9.5	++	++	+++	+++

R1, R2: Growth assessment of replicates 1 and 2, respectively. +++, good; ++, moderately good; +, sparse; –, lack of growth but survival of inoculum.

Table 3. Growth of the three 'freshwater' clones after 21 days under different salinity regimes, assessed visually

Salinity (ppt)	*Nitzschia palea*		*Nitzschia filiformis* var. *conferta*		*Eolimna subminuscula*	
	R1	R2	R1	R2	R1	R2
0	+++	+++	+++	+++	+++	+++
1	+++	+++	+++	+++	+++	+++
6	+++	+++	+++	+++	++ clumpy	++ clumpy
16	++ very clumpy	++ very clumpy	++ protoplast release	++ protoplast release	+	+
22	++ very clumpy	++ very clumpy	++ protoplast release	++ protoplast release	–	–

R1, R2: Growth assessment of replicates 1 and 2, respectively. +++, good; ++, moderately good; +, sparse; –, lack of growth but survival of inoculum.

clone were transferred to each salinity (Tables 2,3) and cultures were grown in illuminated cabinets at 22 °C under a light intensity of 10–15 μmol m^{-2} s^{-1} and a LD (light : dark) cycle of 16 : 8 h lit by cool white fluorescent lights (NHM).

After 21 days of culture, the cells were cleaned by heating with nitric acid. After washing to remove spent acid, frustules were mounted in Naphrax or coated with gold-palladium for examination, using light or scanning electron microscopy, respectively. Because of the small size of the diatoms studied, all the cell measurements were made under a high-resolution field emission scanning electron microscope (Zeiss Ultra Plus, Zeiss, Oberkochen, Germany) (NHM). The variables measured from at least 10 valves in each treatment were: length (μm), width (μm), number of fibulae in 10 μm and number of striae in 10 μm. A qualitative assessment of the growth response of each strain to salinity was made visually at day 21 under an inverted microscope (Olympus CK2, Olympus UK, Watford, UK).

For each species, multivariate ANOVA (MANOVA) was used to test for significant differences in cell morphology between salinity treatments. Statistical analyses were performed using a software package (SPSS for Windows, Release 12.0.0., SPSS Inc., Chicago, IL, USA). The level of statistical significance used was $P < 0.05$.

Table 4. Results of multivariate analysis of variance (MANOVA) of characters against salinity for the five diatom species studied

Diatom species	Variable	d.f.	*F*	*P*
Nitzschia pusilla	**Length**	3, 38	6.455	**0.010**
	Width	3, 38	3.770	**0.016**
	# fibulae/10 μm	3, 38	6.177	**0.002**
	# striae/10 μm	3, 38	3.643	**0.021**
Nitzschia frustulum	Length	3, 41	1.311	0.284
	Width	3, 41	4.302	**0.010**
	# fibulae/10 μm	3, 41	2.149	0.109
	# striae/10 μm	3, 41	5.912	**0.002**
Nitzschia palea	**Length**	3, 40	3.012	**0.041**
	Width	3, 40	4.022	**0.014**
	# fibulae/10 μm	3, 40	2.151	0.109
	# striae/10 μm	3, 40	1.090	0.364
Nitzschia filiformis var conferta	**Length**	3, 41	3.730	**0.018**
	Width	3, 41	64.808	**<0.001**
	# fibulae/10 μm	3, 41	0.179	0.910
	# striae/10 μm	3, 41	0.677	0.571
Eolimna subminuscula	**Length**	3, 40	16.436	**<0.001**
	Width	3, 40	7.894	**<0.001**
	# striae/10 μm	3, 40	0.125	0.945

Degrees of freedom (d.f.), *F*-statistics and *P* values. **Bold** type indicates significant difference with variable ($P < 0.05$). Characters investigated: valve length and width, number of fibulae in 10 μm, number of striae in 10 μm.

RESULTS

Growth at different salinities

Whereas the 'brackish' clones (i.e. *N. pusilla, N. frustulum*) grew well over the entire salinity range, with no or very little change in growth success (Table 2), the three freshwater clones (i.e. *N. palea*, *N. filiformis* var. *conferta, E. subminuscula*) were negatively affected at higher salinities (Table 3). The growth of *E. subminuscula* was already modified at 6 ppt (not only becoming slightly slower but also showing a clumped distribution of cells in the culture), at 16 ppt growth was very slow, and the culture failed to grow at 22 ppt. In contrast, the speed of growth in both *N. palea* and *N. filiformis* var. *conferta* was only slightly affected even at 16 and 22 ppt. However, these salinities caused a change in the spatial distribution of cells of *N. palea* (very clumped) and induced protoplast release in many cells of *N. filiformis* var. *conferta*.

Effects of salinity on valve morphology

Salinity subtly but significantly affected the morphology of the five diatoms. However, the effects were different in the different species.

N. pusilla (Fig. 6): The classical morphological characters (i.e. length, width, fibula density and stria density) of *N. pusilla* were all significantly affected by salinity (Table 4). At the end of the experiment the length of this species was significantly lower at 32 ppt than at the other salinities. Valve width decreased with decreasing salinity. Both fibula and stria densities were lowest at the lowest salinities (Figs 8–11).

N. frustulum (Figs 1,2): Width and stria density were significantly affected by salinity (Table 4). Both were lowest at the highest salinities (Figs 12,13).

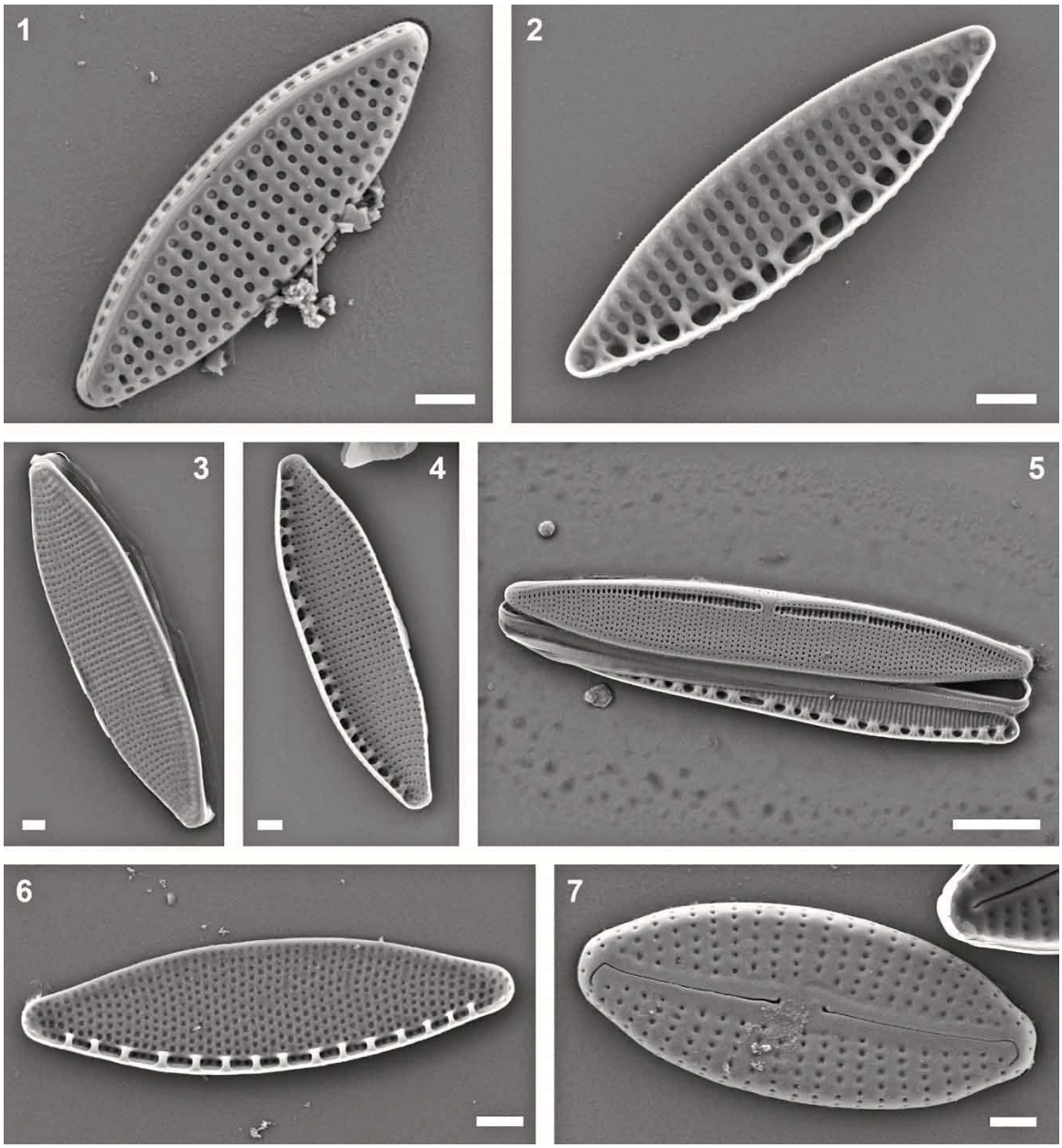
N. palea (Figs 3,4): Length and width were significantly affected by salinity (Table 4) but there was no overall trend, width for example being lowest at 6 ppt (Figs 14,15).

N. filiformis* var. *conferta (Fig. 5): Length and width were again the morphological characters affected by salinity (Table 4). Valve width increased with increasing salinity. The significant change in length (greatest length) was found at 6 ppt (Figs 16,17).

E. subminuscula (Fig. 7): As in the other two 'freshwater' species, length and width were the morphological characters affected by salinity (Table 4). The greatest valve lengths and the lowest valve widths were found at intermediate salinities (1 & 6 ppt) (Figs 18,19).

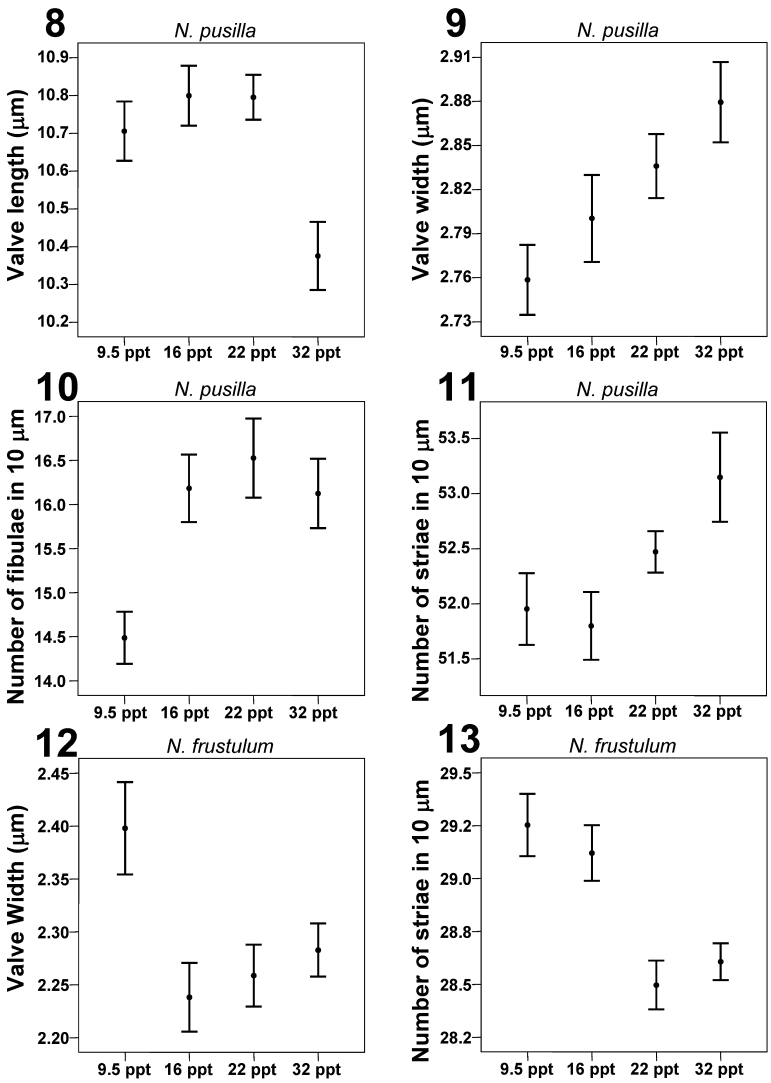
DISCUSSION

Comparatively, the 'brackish' clones (*N. pusilla, N. frustulum*) coped better with low salinities than the 'freshwater' ones (*N. palea*, *N.filiformis* var. *conferta* and *E. subminuscula*) did with high salinities. In an earlier



Figs 1–7. Scanning electron micrographs of the five strains studied. Scale bars represent 1 µm (Figs 1–4,6,7) or 5 µm (Fig. 5). 1,2. External and internal valve views of *Nitzschia frustulum,* respectively. 3,4. Internal and external valve views of *Nitzschia palea,* respectively. 5. External valve view of *Nitzschia filiformis* var. *conferta.* 6. *Nitzschia pusilla* inner valve view. 7. External valve view of *Eolimna subminuscula*.

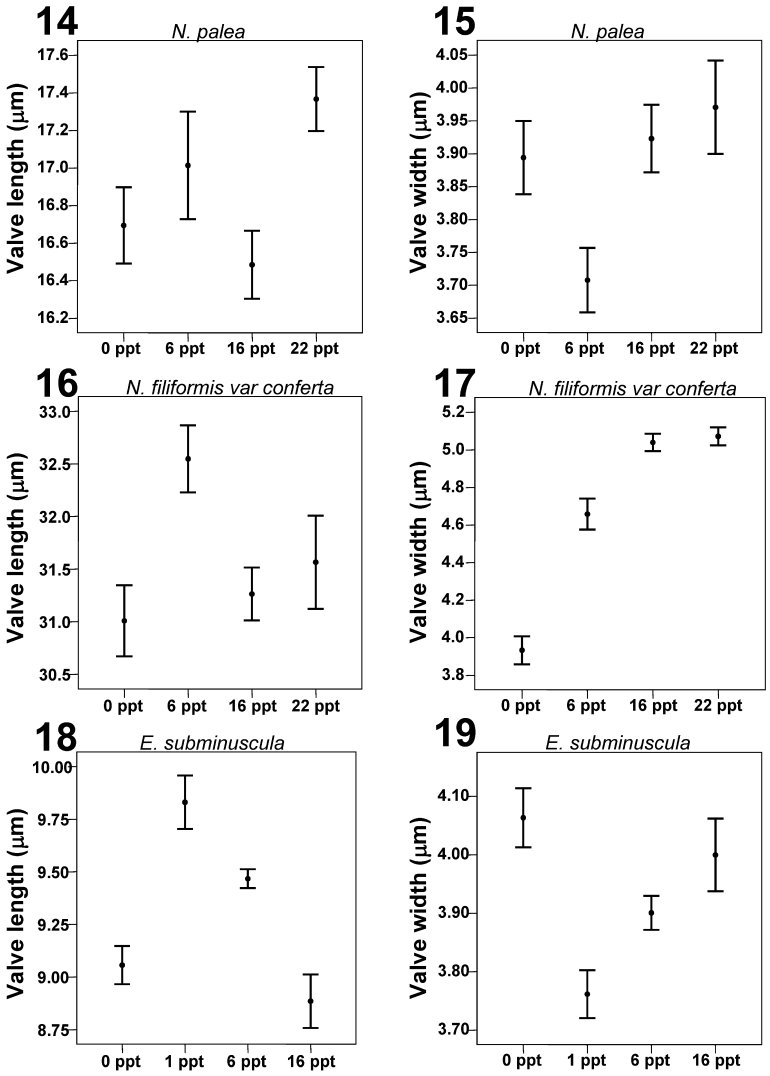
work, Cox (1995) found apparently opposite results when working with four clones of *Navicula*. In her study, the two freshwater clones (*N. gregaria* Donkin, *N. veneta* Kützing) grew well under fully saline conditions, whereas the brackish/marine ones (i.e. *N. phyllepta* Kützing, *N. incertata* Lange-Bertalot) failed to grow in very low salinity or freshwater medium. However, two factors need to be taken into account when interpreting Cox's results and ours. First, in both Cox's (1995) study and ours, clones were classified as 'freshwater' or 'brackish' according to the growth medium in which the clones were originally established, which was in turn guided by



Figs 8–13. Error bar graphs showing the morphological variation of *Nitzschia pusilla* and *Nitzschia frustulum* due to salinity regime. 8. Variation in valve length in *N. pusilla*. 9. Variation in valve width in *N. pusilla*. 10. Variation in fibula density in *N. pusilla*. 11. Variation in stria density in *N. pusilla*. 12. Variation in valve width in *N. frustulum*. 13. Variation in stria density in *N. frustulum*. Only significantly affected morphological characters are shown.

the salinity of the samples from which the clones were isolated. The designations 'freshwater' and 'brackish' may therefore not properly characterize the salinity preferences of the species (as in the halobion classifications of Kolbe 1927, or Hustedt 1953). Second, many taxa from brackish waters seem to be euryhaline (Simonsen 1962; Eppley 1977; Carpelan 1978). Our results show that, although four of the five estuarine species studied here grow well over a broad salinity range, some (*N. pusilla, N. frustulum*) have a broader tolerance to salinity variation (from fully saline down to at least 9.5 ppt; lower salinities were not tested) than others (*N. palea, N.filiformis* var. *conferta* grew less well at 16 ppt and above).

Significant differences in the valve morphology of *N. pusilla*, *N. frustulum*, *N. palea*, *N. filiformis* var. *conferta* and *E. subminuscula* were detected after three weeks growth under different salinity conditions. However, there was no consistent pattern in either the morphological characters affected or the direction of change. Our results confirm and extend the conclusion of Trobajo *et al*. (2004b) that the effects of salinity on pennate diatom morphology are taxon- or perhaps even clone-specific. For instance, Jahn (1986) found that salinity had a clear effect on valve width (reducing it) in clones of *Gomphonema augur* Ehrenberg, but not on stria density. Wendker and Geissler (1988) reported significant differences in length (decrease), width (decrease), stria density (decrease) and fibula density (increase) in clones of *Nitzschia palea* var. *debilis* (Kützing) Grunow grown in enriched chloride-medium (7.5 g Cl^- L^{-1}), and significant differences in length (decrease), width (increase) and fibula density



Figs 14–19. Error bar graphs showing the morphological variation of *Nitzschia palea, Nitzschia filiformis* var. *conferta* and *Eolimna subminuscula* due to salinity regime. 14. Variation in valve length in *N. palea*. 15. Variation in valve width in *N. palea*. 16. Variation in valve length in *N. filiformis* var. *conferta*. 17. Variation in valve width in *N. filiformis* var. *conferta*. 18. Variation in valve length in *Eolimna subminuscula*. 19. Variation in valve width in *E. subminuscula*. Only significantly affected morphological characters are shown.

(decrease) in clones of *Nitzschia gandersheimiensis* Krasske, grown in even more enriched chloride-medium (15 g Cl^{-} L^{-1}). In our data, increasing salinity caused width to increase in *N. pusilla*, as in *N. gandersheimiensis*, but stria density also increased, in contrast with *N. ganderheimiensis,* and also with *G. augur* and *N. palea* var. *debilis*, in which stria density remained constant.

In an earlier study (Trobajo *et al*. 2004b) a *Nitzschia* clone that was identified as *N. frustulum* (following Krammer & Lange-Bertalot 1988) was isolated. This clone showed different morphological responses to salinity than the present *N. frustulum* clone. Length, width and fibula density were the morphological characters significantly affected by salinity in the earlier clone, whereas in the present one the significantly affected morphological characters were width and stria density. However, direct comparison is difficult, because the salinity treatments in the two studies were not the same (0.52, 7.0 and 17.5 ppt in Trobajo *et al*. 2004b; 9.5, 16, 22 and 32 ppt here). Interestingly, for width (the only character affected significantly in both studies) the observed trend is the same over comparable salinities (i.e. 7 vs. 9.5 ppt, 17.5 vs. 16 ppt, previous vs. present, respectively). We are currently investigating whether any differences between the two clones could be caused by genotypic differentiation within a single species, or the presence of several closely related and morphologically similar 'species' within *N. frustulum* (i.e. *N. frustulum* is a 'species complex' containing several pseudocryptic or cryptic species).

Previous studies have suggested that width and/or stria density are particularly reliable taxonomic characters in some *Nitzschia* species (Geissler 1970a,b; Wendker & Geissler 1988; Trobajo *et al*. 2004b, 2006, 2009), as shown by their low intraclonal variation, either when subjected to different conditions, or during the entire life cycle. We found a significant effect of salinity on stria density in *N. frustulum* and in *N. pusilla*, and on width in all five species studied. However, the changes were very small and therefore do not undermine the usefulness of these characters for the discrimination of these *Nitzschia* species. Nevertheless, and to be sure, further experiments over a longer period would be desirable, although the changes observed in valve morphology might then be determined more by intrinsic changes due to the life cycle than to the salinity conditions, as was observed in *N. palea* var. *debilis* and *N. gandersheimiensis* (Wendker & Geissler 1988).

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Chapter 4

Trobajo, R., Rovira, L., Ector, L., Morales, E., Wetzel, C. E., Kelly, M. & Mann, D.G. (2012) **Morphology and identity of some ecologically important small *Nitzschia* species.** Diatom Research 28 (1), 37-59.

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Morphology and identity of some ecologically important small *Nitzschia* species

ROSA TROBAJO[1]*, LAIA ROVIRA[1], LUC ECTOR[2], CARLOS E. WETZEL[2], MARTYN KELLY[3] & DAVID G. MANN[4]

[1]*Aquatic Ecosystems, Institute for Food and Agricultural Research and Technology (IRTA), Crta de Poble Nou Km 5.5, Sant Carles de la Ràpita, Catalonia, Spain*
[2]*Public Research Centre – Gabriel Lippmann, Department of Environment and Agro-biotechnologies (EVA), 41 rue du Brill, 4422 Belvaux, Grand-duchy of Luxembourg*
[3]*Bowburn Consultancy, 11 Monteigne Drive, Bowburn, Durham, DH6 5QB, UK*
[4]*Royal Botanic Garden Edinburgh, Edinburgh, EH3 5LR, Scotland*

The taxonomy of several small-celled, ecologically significant *Nitzschia* species, which are frequently confused with each other or whose names are misapplied, is clarified. Following an examination of type material and modern samples by light and electron microscopy, it was concluded that *N. frustulum* (Kützing) Grunow, *N. inconspicua* Grunow, *N. soratensis* E. Morales & Vis and *N. invisitata* Hustedt are independent species. No morphological basis was found for separating *N. frustulum* var. *subsalina* Hustedt or *N. boliviana* E. Morales & Vis from *N. inconspicua* and they are therefore placed in synonymy with *N. inconspicua*. *Nitzschia soratensis*, described recently from Bolivia, has previously been misidentified in Europe, either as *N. inconspicua* (from which it differs most obviously in having more bluntly rounded poles, striae within the raphe canal that are composed of triplets, and fibulae that can be seen in light microscopy to widen at their bases) or as *N. abbreviata* Hustedt ex Simonsen (from which it differs in pore ultrastructure). *Nitzschia frustulum* resembles *N. inconspicua* in every morphological feature examined, but with wider valves and consistently higher maximum length.

Keywords: *diatoms, frustule ultrastructure,* Nitzschia, Nitzschia frustulum, Nitzschia inconspicua, Nitzschia invisitata, Nitzschia soratensis, *taxonomy, type material*

Introduction

Nitzschia Hassall is a widely distributed diatom genus with a large number of species, several of them ecologically important: they are very common (often the most abundant taxa) in different types of inland, coastal and marine waters (references in Trobajo et al. 2004a). Several *Nitzschia* species are good indicators of heavy metal contamination (e.g., Guasch et al. 2009, Tlili et al. 2011), eutrophic and/or organic conditions (e.g., Lange-Bertalot 1979, Kobayasi & Mayama 1982, Van Dam et al. 1994, Martín et al. 2010) and salinity (e.g., Hofmann 1997, Rimet 2009). However, *Nitzschia* is also known for its taxonomic difficulty. One problem is the size of the genus. In 1978, VanLandingham listed ca. 940 species names (both 'acceptable' and 'unacceptable': Mann 1986) and since then a further 170 species names have been added (Fourtanier & Kociolek 2011). Besides these, there are hundreds of named varieties and forms, whose status has mostly not been checked. The type specimens of many taxa have not been studied in detail or were never designated. Moreover, *Nitzschia* species offer few characters for diagnosis under the light microscope (LM): they have finely structured valves with parallel striae, limited variation in valve outline (usually narrowly lanceolate valves with slightly protracted or subcapitate poles) and the raphe is almost invisible. Consequently, identification of cleaned valves is often difficult and the taxonomy of *Nitzschia* is unstable. Identification of living cells is even more problematic, since the chloroplast arrangement is almost constant, with two elongated plates lying end-to-end along the cell, which obscure the little detail present on the valves. *Nitzschia* section Lanceolatae Grunow, which includes the species treated in this study, is particularly challenging, since most have small–medium cells (ca. 5–40 μm length × 2–6 μm width) with very delicate structure (rarely less than 20 striae in 10 μm).

Nevertheless, there are reasons for optimism about improving the classification and identification of *Nitzschia* species. Many types and other specimens have been examined and documented by electron microscopy (e.g., Archibald 1966, 1972a, b, Lange-Bertalot 1977, 1978, 1980, 1990, Lange-Bertalot & Simonsen 1978, Coste & Ricard 1980, 1981, Archibald 1983, Kobayasi 1985, Lobo & Kobayasi 1990, Lobo et al. 1990, Medlin & Hasle 1990, Denys & Lange-Bertalot 1998, Morales & Hamilton 2002, Genkal & Popovskaya 2003, Trobajo & Cox 2006, Tudesque et al. 2008, Alakananda et al. 2012,

*Corresponding author. Email: rosa.trobajo@irta.cat

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Cocquyt et al. 2012, Trobajo et al. 2012), and useful extra characters have been found, including aspects of areola structure, the presence or absence of a marginal ridge, raphe endings, fibula morphology, and girdle structure (e.g., Mann 1978, 1986). Furthermore, some experimental work has been carried out to study the effects of environmental conditions on the valve morphology and growth of *Nitzschia* species (Trobajo et al. 2004a, 2011), and various molecular markers have been evaluated for their potential to resolve species boundaries and relationships in *Nitzschia* and *Pseudo-nitzschia* H. Peragallo (e.g., Amato et al. 2007, Trobajo et al. 2009, 2010, Rimet et al. 2011).

The status of five problematic taxa of *Nitzschia* sect. Lanceolatae is evaluated. All five taxa have very small cells and simple elliptical to lanceolate outlines and their classification has been in flux for more than 30 years. However, despite their confused taxonomy, the supposed ecology of these taxa is routinely used to assess and monitor water quality and ecosystem health (Van Dam et al. 1994, Trobajo et al. 2004b, Gomà et al. 2005, Potapova & Charles 2007, Tornés et al. 2007, Rovira et al. 2009, 2012a), or used to infer past salinity or nutrient conditions (Ryner et al. 2007, Hassan et al. 2009, Hadley et al. 2010).

Nitzschia frustulum (Kützing) Grunow was initially described as *Synedra frustulum* by Kützing (1844) and the type material has been examined in detail by Lange-Bertalot (1977) and Trobajo & Cox (2006). Like many other long-established diatom species, *N. frustulum* accumulated infraspecific taxa between 1844 and the 1980s, with over 30 varieties and forms listed by Fourtanier & Kociolek (2011). Very few of these are used and recorded, the main exceptions being *N. frustulum* var. *perpusilla* (Rabenhorst) Van Heurck, var. *perminuta* Grunow and var. *subsalina* Hustedt, because these were the three infraspecific taxa included in the standard flora used between 1930 and 1988 for identifying freshwater diatoms (Hustedt 1930). *Nitzschia frustulum* var. *perminuta* is now treated as an independent species, *N. perminuta* (Grunow) M. Peragallo (Krammer & Lange-Bertalot 1988), and is not considered here. *Nitzschia frustulum* var. *perpusilla* was discussed by Lange-Bertalot (1977), who disagreed with Hustedt's (1930) opinion that it was synonymous with *N. inconspicua* Grunow, first described for a particularly small-celled *Nitzschia* by Grunow (1862). The identity of *N. inconspicua* remains unclear and conflicting opinions exist concerning the relationships between this species and *N. frustulum* var. *subsalina*, *N. abbreviata* Hustedt ex Simonsen and *N. invisitata* Hustedt; different classifications and names are offered by Lange-Bertalot (1977, 1993), Lange-Bertalot & Simonsen (1978), Krammer & Lange-Bertalot (1988) and Hofmann et al. (2011).

Here, an attempt is to clarify the taxonomy of these diatoms using light and electron microscopy, with special reference to type material, with the aim of providing clear criteria for their identification and a sound taxonomy as a basis for further biogeographical and ecological work.

Material and methods

Slides and mounted type material of *N. frustulum* var. *frustulum*, *N. frustulum* var. *subsalina*, *N. inconspicua* and *N. invisitata* were examined using light and scanning electron microscopy (SEM). Recently, Morales & Vis (2007) described two new related species from the South American Bolivian Altiplano: *N. soratensis* E. Morales & Vis and *N. boliviana* E. Morales & Vis; type materials of both species were here also illustrated and described in detail. Additionally, modern samples from the UK and Spain were also examined under LM and SEM.

The following materials were studied:

(1) *Nitzschia frustulum* var. *frustulum*: some new illustrations were prepared from the type material specified and examined by Trobajo & Cox (2006).
(2) *Nitzschia frustulum* var. *subsalina*: Hustedt Collection, slide 144/1 Oldesloe, Holstein I, 1; 15 July 1922, and unmounted material E3687 (Hustedt Collection at Bremerhaven [BRM]). Unmounted material was prepared for SEM and a new slide was prepared in Naphrax (Brunel Microscopes: http://www.brunelmicroscopes.co.uk/) from the unmounted material and this is preserved in the Royal Botanic Garden Edinburgh (E) as E5159/1.
(3) *Nitzschia inconspicua*: the original slide 372 in the Grunow Collection in the Naturhistorisches Museum Wien was studied. Some unmounted material (Grunow Collection Acquisition 1901, no. 935) was prepared for SEM and also a new slide prepared in Naphrax as E5154 (E).
(4) Specimens of *N. inconspicua* were also studied from the following modern samples:
   - River Idle, Tiln, Nottinghamshire (UK National Grid Reference [NGR]: SK 714 857). Sample collected on 16 July 2001, from submerged cobbles (sample MK101123);
   - River Erewash, Toton, Nottinghamshire (UK NGR: SK 503 342). Sample collected on 6 September 2001, from submerged cobbles (sample MK101187);
   - Clumber Lake, Nottinghamshire (UK NGR: SK 624 760). Sample collected on 30 July 2001, substrate unknown (sample MK102015);
   - Four sites from the Ebro Estuary (Catalonia, Spain, 40°43′16.59″N, 0°40′37.79″E): (i) E4 site (18 km up from river mouth). Sample collected from artificial substrata (fired clay bricks) at a deep level (4 m). Collected on 22 April 2008. (ii) E5 Site (12 km up from river mouth). Sample collected from artificial substrata (fired clay bricks) at a deep level (6.1 m). Collected on 15 December 2008. (iii) E5 Site (12 km up from river mouth).

Sample collected from artificial substrata (fired clay bricks) at a surficial level (0.5 m). Collected on 8 July 2008. (iv) E7 Site (3.5 km up from river mouth). Sample collected from wood debris at a surficial level. Collected on 22 April 2008.

(5) *Nitzschia invisitata*: Hustedt Collection, slide M1/69, 'Celebes 71, Mahalone-See', and unmounted material AS1322 (BRM).

(6) *Nitzschia* sp. 1: In some modern samples from UK freshwater sites valves were found in LM that were very similar to those of *N. inconspicua* and had been identified under this name, but which had a distinct ultrastructure. Individuals from the following samples were used for morphometrics of *Nitzschia* sp. 1. (i) River Yarrow, downstream Croston Sewage Treatment Works (STW), Lancashire (UK NGR: SD 478 187). Collected on 26 August 1997, from submerged cobbles (sample MK98050). (ii) Yarty River, Gammons Hill, downstream A35, Devon (UK NGR: SY 282 983). Collected on 13 August 2002, from submerged cobbles (sample MK102240). (iii) Houselop Beck, Bradley, County Durham (UK NGR: NZ 106 362). Collected on 11 June 2009, from submerged cobbles.
Light micrographs were prepared from the River Irwell upstream, Bolton STW (UK NGR: SE 763049; slide MK100518). SEM observations were made on clonal material (clones NIT1008KEL and NIT1009KEL) isolated from submerged cobbles in Houselop Beck (see above) in June 2011.

(7) *Nitzschia soratensis* and *N. boliviana*: LM and SEM pictures were made from the oxidized holotype material (epilithon), collected from a stream at the Alpine region of Sorata (Stream no. 27, 15°46.874′S, 68°39.677′W, in McClintic et al. 2003), Department of La Paz, Bolivia, and available at the Academy of Natural Sciences of Philadelphia Diatom Collection (ANSP GC 26804).

Modern samples were cleaned with 70% nitric acid and rinsed (by decanting) with deionized water until neutral pH was reached.

LM observations and morphometric measurements were performed with a Zeiss Axio Imager M2 using a Plan-Apochromat × 100 objective (numerical aperture: 1.4) with bright field and differential interference contrast optics; photographs were taken using an Axiocam HRc digital camera. Where it was important to obtain maximum resolution, especially to check the visibility of valve poroids in LM, the condenser was oiled. For SEM examination of *N. frustulum*, *N. frustulum* var. *subsalina*, *N. inconspicua* and *Nitzschia* sp. 1, the cleaned frustules were dried onto glass coverslips attached to aluminium stubs, coated with gold–palladium or platinum, and studied in a Zeiss Ultra Plus or LEO Supra 55VP. Type material of each of *N. invisitata*, *N. soratensis* and *N. boliviana* was concentrated on a polycarbonate membrane filter with a 3-μm mesh, attached to aluminium stubs and sputtered with a 30-nm platinum layer. The stubs were then examined out with a Hitachi SU-70 field emission scanning electron microscope using an accelerated voltage of 5 kV.

Significant differences in cell morphology were tested with analysis of variance (ANOVA), followed by Games–Howell post-hoc tests. These analyses were performed with SPSS v 12.0 and the level of statistical significance used was $p < 0.05$.

Results

Nitzschia frustulum *(Kützing) Grunow in Cleve & Grunow 1880 var.* frustulum *(Figs 1–9, 45–46, 55–60)*

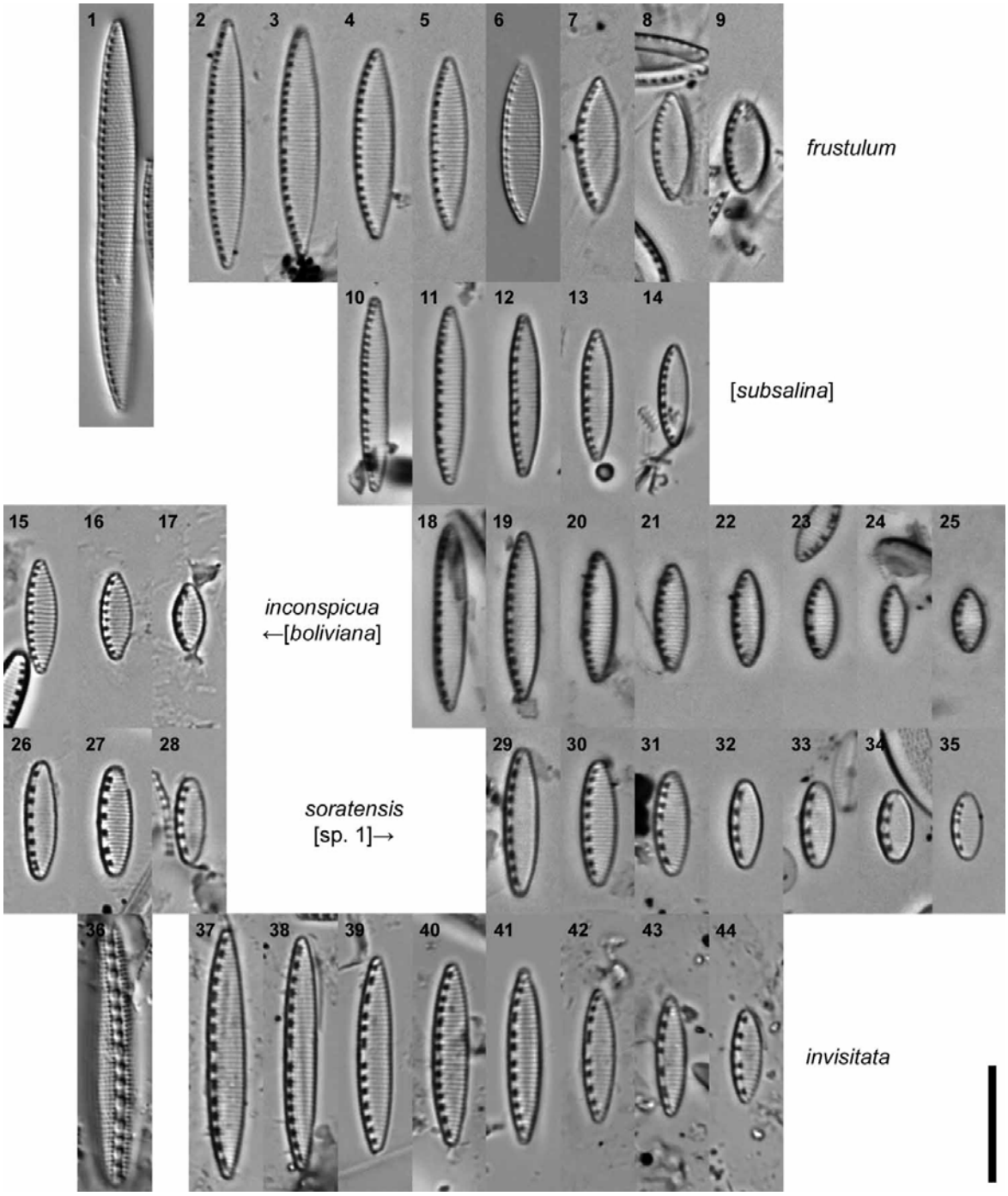
Basionym. *Synedra frustulum* Kützing 1844, p. 63, pl. 30, fig. 77.

Light and electron microscopy of type material. Additional new LM and SEM micrographs are given along with data derived from Trobajo & Cox (2006) to facilitate comparisons with *N. frustulum* var. *subsalina*, *N. inconspicua* and the other species for which new information is presented (Table 1). The type slide was also scanned for initial cells (i.e., the first cell formed within the auxospore). Although none were found, two long valves measuring 33 and 34 μm (e.g., Fig. 1) were found that were slightly flexed, as might be expected in post-initial cells.

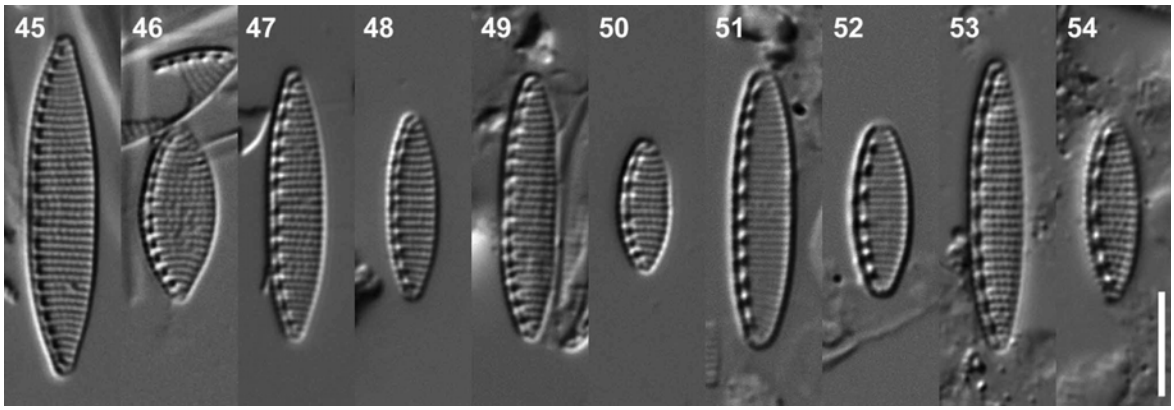
The valves of *N. frustulum* var. *frustulum* are linear to lanceolate, with slightly protracted, narrow apices (Figs 1–9, 45–46). The striae are uniseriate, both on the valve face and within the raphe canal (Figs 57–60), where each stria is represented by a single areola (Figs 58–59). The boundary between the raphe canal and the valve face is not marked externally by a wider separation of the stria areolae (Figs 58–59). On the mantle, the striae are very short, comprising one areola within the raphe canal and one outside (Fig. 58). All areolae are round and occluded by a delicate hymen, which can be detected both externally and internally (Figs 58–60 and see Trobajo & Cox 2006). The raphe is interrupted centrally by a small central nodule (Fig. 60), the external central raphe endings are straight and slightly depressed (Fig. 58) and the polar raphe endings are strongly hooked towards either the valve face (Fig. 59) or the mantle (Trobajo & Cox 2006). The fibulae are short bars, whose bases are linked by slight longitudinal ridges of silica (Fig. 60).

Nitzschia frustulum *var.* subsalina *Hustedt 1925 (Figs 10–14, 47–48, 61–69)*

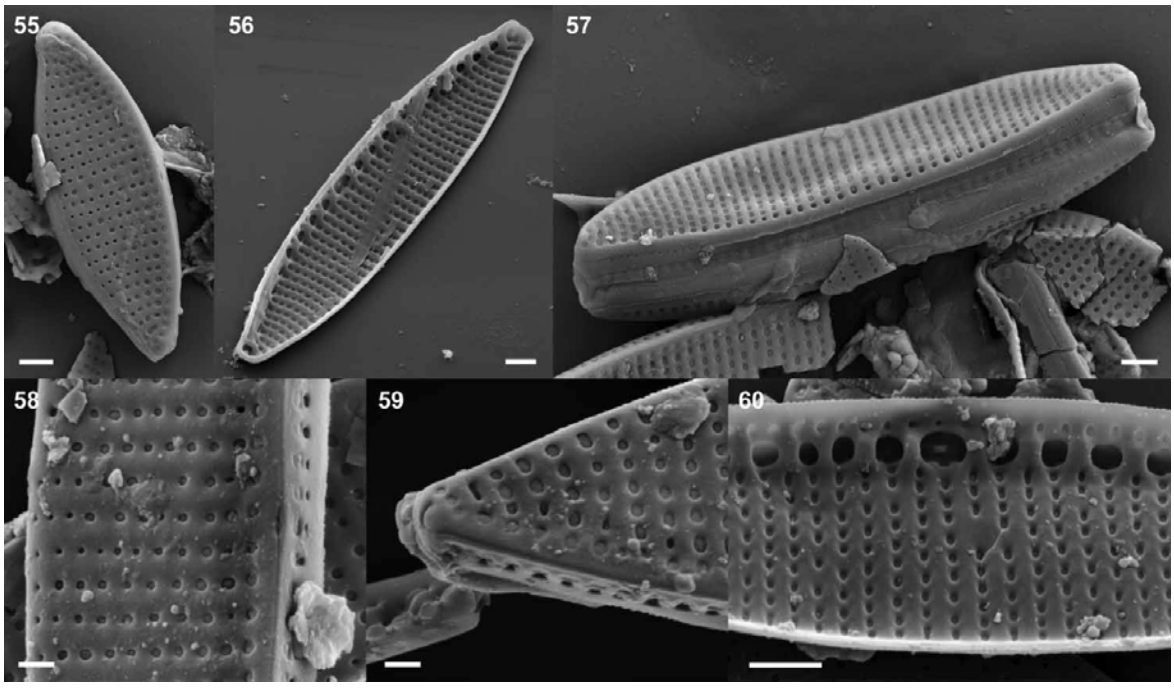
Hustedt (1925) described var. *subsalina* from brackish water sites near Oldesloe, Schleswig-Holstein, Germany, where he found it as isolated individuals ('vereinzelt') in three sets of samples. Although the taxon was not abundant, it was not difficult identifying specimens on slide 144/1



Figs 1–44. Light micrographs in bright field optics showing the size variation in five *Nitzschia* taxa, with valves of almost equal length in vertical columns (except Figs 1, 15–17, 26–28 and 36) in order to facilitate inter-species comparisons. **Figs 1–9.** *Nitzschia frustulum*, slide BM18159, the lectotype slide of '*Synedra frustulum*' comprising material from Meneghini, Kützing accession 210. Figure 1 is the longest valve discovered on the type slide. **Figs 10–14.** Type material of *N. frustulum* var. *subsalina* on slide E5159/1. **Figs 15–17.** Type material of *N*. *boliviana* from Sorata Department, Bolivia, ANSP GC 26804 (= *N. inconspicua*). **Figs 18–25.** Type material of *N*. *inconspicua* on slide E5154. **Figs 26–28.** Type material of *N*. *soratensis* from Sorata Department, Bolivia, ANSP GC 26804. **Figs 29–35.** *Nitzschia* sp. 1 (= *N. soratensis*) on slide MK100518 (at E) from the River Irwell. **Figs 36–44.** *Nitzschia invisitata*, isolectotype material on slide M1/69, Hustedt Collection. **Fig. 36.** Initial valve. Scale bar = 10 μm.



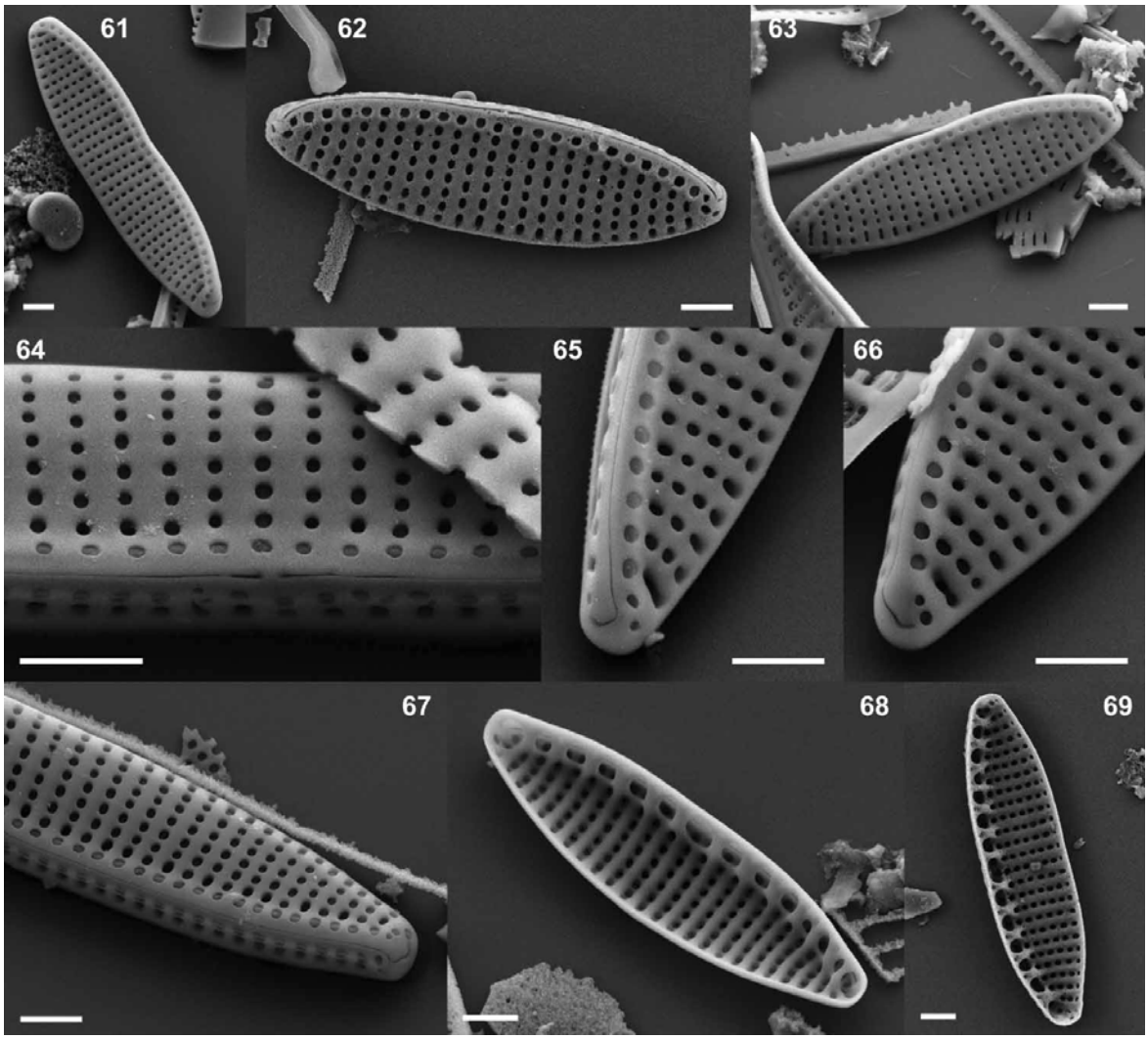
Figs 45–54. Selected valves of five *Nitzschia* taxa in differential interference contrast optics showing stria arrangement and structure. **Figs 45–46.** *Nitzschia frustulum* var. *frustulum*. **Figs 47–48.** *Nitzschia frustulum* var. *subsalina* (= *N. inconspicua*). **Figs 49–50.** *Nitzschia inconspicua*. **Figs 51–52.** *Nitzschia* sp. 1 (= *N. soratensis*). **Figs 53–54.** *Nitzschia invisitata*. Scale bar = 5 μm.



Figs 55–60. *Nitzschia frustulum* var. *frustulum* from type material, Kützing accession 210, external (Figs 55, 57–59) and internal (Figs 56, 60) views. **Fig. 55.** Whole valve. **Fig. 56.** Whole valve. **Fig. 57.** Frustule in girdle view, note the lack of a discrete, separated row of areolae on the mantle. **Fig. 58.** Central raphe endings. **Fig. 59.** Hooked terminal raphe fissure. **Fig. 60.** Fibulae and central nodule. Scale bars = 1 μm.

that agreed with the typification by Simonsen (1987, p. 93, pl. 132, figs 4–10).

Light microscopy of type material. Variation in valve dimensions and features of specimens found on the type slide and corresponding to *N. frustulum* var. *subsalina* are given in Table 1. The valves were linear, becoming lanceolate in the shortest specimens (Figs 10–14); no individuals shorter than 8 μm were found on the type slide or the new slide. All the specimens had slightly protracted, narrow apices, but these became less obvious in smaller specimens. The fibulae were quite regularly distributed along the raphe canal and shaped like small rectangular dots, with the two median ones usually further apart than the others (Figs 11–14, 47–48). However, in some valves the median interspace was of the same width as the other interspaces (Fig. 10). The striae were very conspicuous and parallel throughout most of the valve, becoming slightly radiate towards the apices; the areolae can be resolved in LM (Figs 47–48).



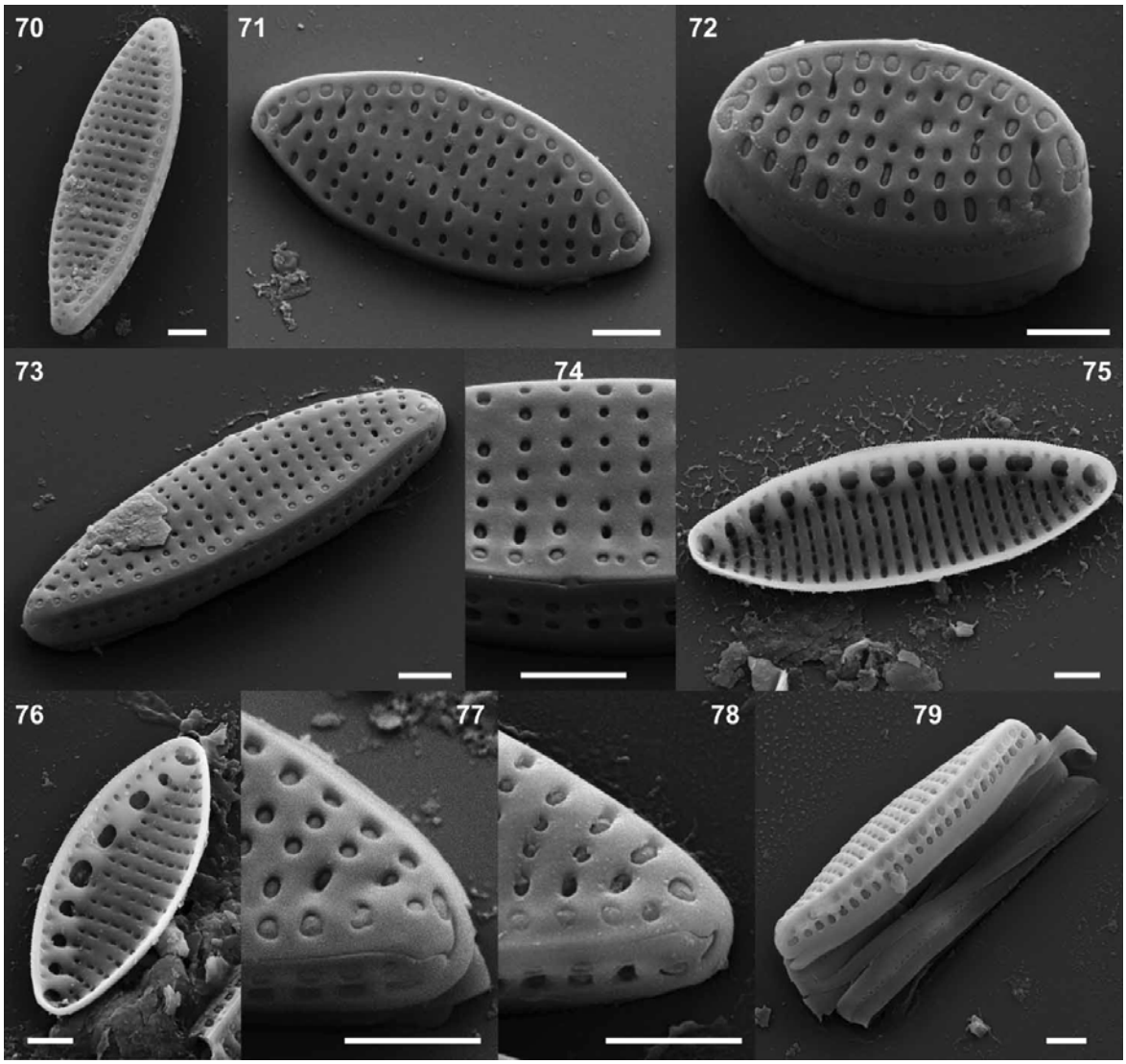
Figs 61–69. *Nitzschia frustulum* var. *subsalina*: type material (accession E3687 at BRM), external (Figs 61–67) and internal (Figs 68–69) views. **Fig. 61**. Whole valve: note the central constriction of the raphe canal. **Fig. 62.** Whole valve: eroded specimen. **Fig. 63**. Valve seen from the side opposite the raphe: note the lack of a discrete, separated row of areolae near the edge of the valve (contrast *N. soratensis*, Fig. 86). **Fig. 64**. Central raphe endings; note also the hymenes in the areolae of the raphe canal. **Figs 65–66**. Terminal fissures turned towards the mantle or valve face. **Fig. 67**. Valve pole, showing raphe canal areolae containing hymenes and kinked and hooked terminal raphe fissure. **Figs 68–69**. Valves showing fibulae. Scale bars = 1 μm.

Electron microscopy of type material. The type material of *N. frustulum* var. *subsalina* appeared to have suffered some dissolution, judging by the porous appearance of the silica at high magnifications in many valves (e.g., Figs 62, 69). As in the nominate variety, the striae were uniseriate, both on the valve face and within the raphe canal, and the boundary between the raphe canal and the valve face was not marked externally by a wider separation of the stria areolae (Figs 61–67). Internally, hymenes could be detected only in a few, apparently better-preserved valves (torn hymenes were visible in the valve shown in Fig. 68); externally, hymenes were often detected in the row of areolae bordering the raphe, within the raphe canal (Figs 63–65, 67; contrast the eroded valve in Fig. 62) but rarely elsewhere. The raphe was interrupted centrally by a small central nodule. The external central endings were straight (Figs 62, 64) and usually (Figs 61, 63), but not always (Fig. 62), somewhat depressed. The terminal raphe endings were hooked towards either the mantle (Fig. 65) or the valve face (Figs 66–67), often with a backward deflection before the hook (especially obvious in Fig. 67). The fibulae were like those of var. *frustulum* (Figs 68–69).

Table 1. Morphological and morphometric characteristics of eight small-sized *Nitzschia* species from type material and modern collections.

Taxon	Reassigned identity	Valve shape		Apex shape		Valve length (μm)	Valve width (μm)	Striae in 10 μm	Fibulae in 10 μm	Fibula shape	*n*
		Long cells	Short cells*	Long cells	Short cells*						
N. frustulum var. *frustulum* (type material)	*N. frustulum*	Linear–lanceolate to lanceolate	Elliptical–lanceolate	Very slightly protracted, narrow	Very slightly protracted, narrow	18.5 (10.8–34.0)	3.5 (3.0–3.9)	27.6 (26.6–30.3)	14.0 (13.3–15.0)	Small rectangular dots	35
N. frustulum var. *subsalina* (type material)	*N. inconspicua*	Linear to lanceolate	n.d.	Very slightly protracted, narrow	n.d.	12.4 (8.0–16.8)	2.6 (2.4–2.9)	26.1 (25.1–27.8)	13.0 (10.1–15.0)	Small rectangular dots	52
N. inconspicua (type material)	*N. inconspicua*	Linear to lanceolate	Elliptical–lanceolate	Very slightly protracted, narrow	Very slightly protracted, narrow; or not protracted at all	7.5 (6.0–11.5)	2.9 (2.6–3.1)	26.9 (23.7–28.7)	14.0 (10.6–17.0)	Small rectangular dots	20
N. inconspicua (type material + modern samples)	*N. inconspicua*	Linear to lanceolate	Elliptical–lanceolate	Very slightly protracted, narrow	Very slightly protracted, narrow; or not protracted at all	7.0 (4.1–15.3)	2.7 (2.3–3.1)	26.8 (23.7–30.4)	13.0 (8.9–17.0)	Small rectangular dots	266
N. boliviana (type material)	*N. inconspicua*	Linear to lanceolate	Elliptical–lanceolate	Very slightly protracted, narrow	Very slightly protracted, narrow	8.0 (6.0–9.9)	2.9 (2.6–3.2)	26.0 (24.1–27.1)	12.6 (10.9–13.6)	Small rectangular dots	17
Nitzschia sp. 1 (modern samples)	*N. soratensis*	Linear–lanceolate to lanceolate	Elliptical–lanceolate to elliptical	Very slightly protracted, broad	Very slightly protracted, broad; or not protracted at all	7.8 (4.7–13.7)	2.8 (2.3–3.3)	29.1 (27.5–30.7)	10.8 (7.0–15.0)	'torn notebook'	73
N. soratensis (type material)[1]	*N. soratensis*	Linear–lanceolate to lanceolate	Elliptical–lanceolate to elliptical	Very slightly protracted, broad	Very slightly protracted, broad; or not protracted at all	9.6 (6.8–13.7)	3.0 (2.6–3.2)	27.9 (27.1–28.7)	10.2 (7.9–13.8)	'torn notebook'	12
N. invisitata (type material)	*N. invisitata*	Linear–lanceolate to lanceolate	n.d.	Very slightly protracted, narrow	n.d.	14.2 (8.3–24.0)	2.9 (2.5–3.5)	24.4 (23.4–25.5)	9.0 (7.0–11.5)	'torn notebook'	39

Note: Mean (minimum–maximum) values are presented for *n* specimens measured. *, cell length <8 μm; [1], measurements of *N. soratensis* type material given by Morales & Vis (2007) were 5.6–15.6 × 2.4–3.2 μm; 27.5–30 striae and 10–12.5 fibulae in 10 μm; n.d., no short cells were found in the material studied.



Figs 70–79. *Nitzschia inconspicua*: type material (from the Raaber Bahnhof, Grunow Collection Acquisition 1901, no. 935), external (Figs 70–74, 77–79) and internal (Figs 75–76) views. **Figs 70–72**. Medium, small and very small valves. Note the clearly visible hymenes in the raphe canal areolae of all three valves, and in the stria areolae of Fig. 72. **Fig. 73**. Whole valve; note that the 'central' raphe endings of this valve are slightly closer to the left-hand pole. **Fig. 74**. Slightly depressed central raphe endings. **Figs 75–76**. Valve interiors. **Figs 77–78**. Kinked and hooked terminal fissures turned either towards the valve face (Fig. 77) or towards the mantle (Fig. 78). **Fig. 79**. Oblique view of disrupted frustule, showing the single row of areolae on the raphe mantle and open girdle bands. Scale bars = 1 μm.

Nitzschia inconspicua ***Grunow 1862 (Figs 18–25, 49–50, 70–79)***

Synonym. *Nitzschia frustulum* var. *inconspicua* (Grunow) Grunow sensu auct. nonnull.

This species was described by Grunow (1862), who specified a single collection from the Raaber Bahnhof in Vienna (the terminus, now demolished, of the old East Railway), made on 14 March 1858 from among green algae on rotting plant material in a warm-water pond.

Diatoms corresponding to Grunow's (1862) description and figures are abundant in the Raaber Bahnhof sample and Grunow later used the same sample to illustrate *N. inconspicua* for Van Heurck's Synopsis (1881, pl. 69, fig. 6). However, as well as the diatom illustrated by Grunow in 1862 (pl. 18, though it is pl. 12 in Grunow's numbering, fig. 25a-e) and described (p. 579) as being 7.7–10 μm long (0.0003–0.0004 Austrian inches) and with 'valvis late ovato lanceolatis subacuminatis', the Raaber material also

contains a different, larger *Nitzschia* species with rostrate apices. This was illustrated alongside the smaller species by Grunow himself (in Van Heurck 1881, the bottom right valve in fig. 6): Grunow's annotated copy of Van Heurck's Synopsis and original drawings in Vienna prove that fig. 6 was drawn from the Raaber material. The larger *Nitzschia* has been illustrated also by Lange-Bertalot (1977, figs 26, 27b) in photographs from Grunow's slide 372, but it is clearly not part of the life-cycle variation of *N. inconspicua* and so is omitted here.

Note: the name *Nitzschia frustulum* var. *inconspicua* (Grunow) Grunow appears to be invalid under Article 34 of the International Code of Botanical Nomenclature (McNeill et al. 2006), because varietal and species status were used simultaneously by Grunow in Van Heurck (1881, pl. 69, fig. 6), where he referred only to '*N*. (*frustulum* var.) *inconspicua* Grun.', without indication that varietal status was to be accepted or preferred. In the text of the Van Heurck Synopsis (1885), neither *N. inconspicua* nor *N. frustulum* var. *inconspicua* was mentioned. In the index to the plates (Van Heurck 1884, p. 72), both are listed, without indication that var. *inconspicua* is to be accepted in preference. *Nitzschia inconspicua* was listed as an independent species without synonyms by Grunow himself (in Cleve & Grunow (1880) and Schönfeldt (1907). In his compendium of published diatom taxa, De Toni (1892) also listed *N. inconspicua* as an independent species but did not list *N. frustulum* var. *inconspicua* as a synonym, contrasting with his treatment of '*N. palea* var.? *romana* Grunow' (from Cleve & Grunow 1880), which was given as a synonym of *Nitzschia romana* Grunow in Van Heurck (1881, where *N. romana* is unambiguously treated as an independent species). No valid publication of *N. frustulum* var. *inconspicua*, based on *N. inconspicua* Grunow, that predates Hustedt's (1925) description of *N. frustulum* var. *subsalina* has been found.

Light microscopy of type material. The variations in valve length and width, stria and fibula density of *N. inconspicua* specimens found in the type slide are given in Table 1. Valve shape varied from linear–lanceolate to lanceolate in longer specimens (Figs 18–19) to elliptical–lanceolate in the shorter ones (Figs 23–25). Most specimens had very slightly protracted, narrow apices, although in the very short specimens, the apices tended to become rounded (Fig. 25). The fibulae were quite regularly distributed along the raphe canal (Figs 18–25, 49–50) and appeared like small rectangular dots, with the two median ones usually further apart than the others. However, in some valves (e.g., Figs 21–22) the median interspace was the same width as the other interspaces; in other cases (not frequently), the wider interspace containing the central raphe endings was displaced towards one end of the cell (not shown in LM but illustrated in SEM, see Fig. 73). The striae were conspicuous and areolae could be resolved within them (Figs 49–50); except in the smallest valves, the striae were parallel throughout most of the valve, becoming slightly radiate towards the apices (i.e., curved margins). In very small specimens almost no parallel striae were present, all being curved (Fig. 25, see also Fig. 72).

Electron microscopy of type material. The ultrastructure of *N. inconspicua* specimens was identical to those of *N. frustulum* var. *subsalina* (e.g., compare Figs 70–73 with Figs 61–63 and Figs 75–76 with Figs 68–69), except that hymenes could be detected more often in *N. inconspicua* specimens from the type material (e.g., Figs 71–74, 77–78), which was probably due to their better preservation. The central external raphe endings were straight (Fig. 74) and slightly depressed (Figs 71, 73–74) and the terminal fissures were hooked to either side, often with a backward deflection as in some var. *subsalina* (Figs 77–78). The oblique view in Fig. 79 illustrates the mantle structure present in *N. inconspicua*, which coincides with that of *N. frustulum* var. *frustulum* and var. *subsalina*, and which consists of a single row of elliptical areolae below the raphe canal. Three or four girdle bands are present in the epitheca, the valvocopula bearing a single row of areolae in the pars exterior, near its junction with the pars interior (Fig. 79).

Modern samples. Nitzschia inconspicua specimens from modern samples showed the same ultrastructure (Supplementary plate 1, Figs 11–14), valve outline (Supplementary plate 1, Figs 1–10), and morphometrics (Table 1) as in the type material, with slight expansion of the ranges of length, stria density and fibula density.

Nitzschia boliviana *E. Morales & Vis 2007 (Figs 15–17, 80–83, supplementary plate 2)*

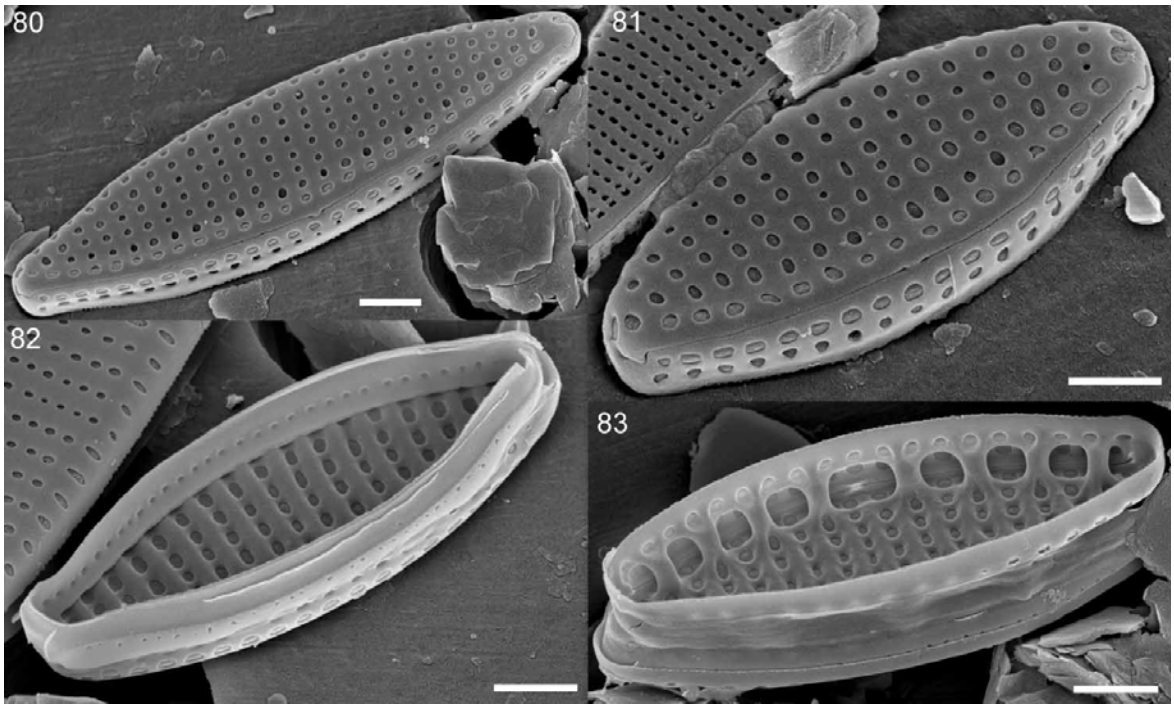
Original description: Morales & Vis 2007, p. 127, figs 260–264, 271–276.

Light and electron microscopy of type material. Selected valves of *N. boliviana* from type material are shown in Figs 15–17. Valve outlines and metrics fell within the ranges exhibited by *N. inconspicua* (Figs 18–25, Table 1). The fibulae were small rectangular dots, the central pair usually being more distantly spaced than the remainder.

The valve and raphe structure were exactly as in *N. inconspicua* and *N. frustulum* var. *subsalina*, including single areolae within the raphe canal and simple central raphe endings (Figs 80–81), a backward deflection of the terminal fissures (Fig. 81), and hymenes that are particularly obvious externally in the areolae adjacent to the raphe (Fig. 81). The girdle bands are open (Fig. 82) and the valvocopula bears a single row of areolae.

Nitzschia *sp. 1 (Figs 29–35, 51–52, 84–96)*

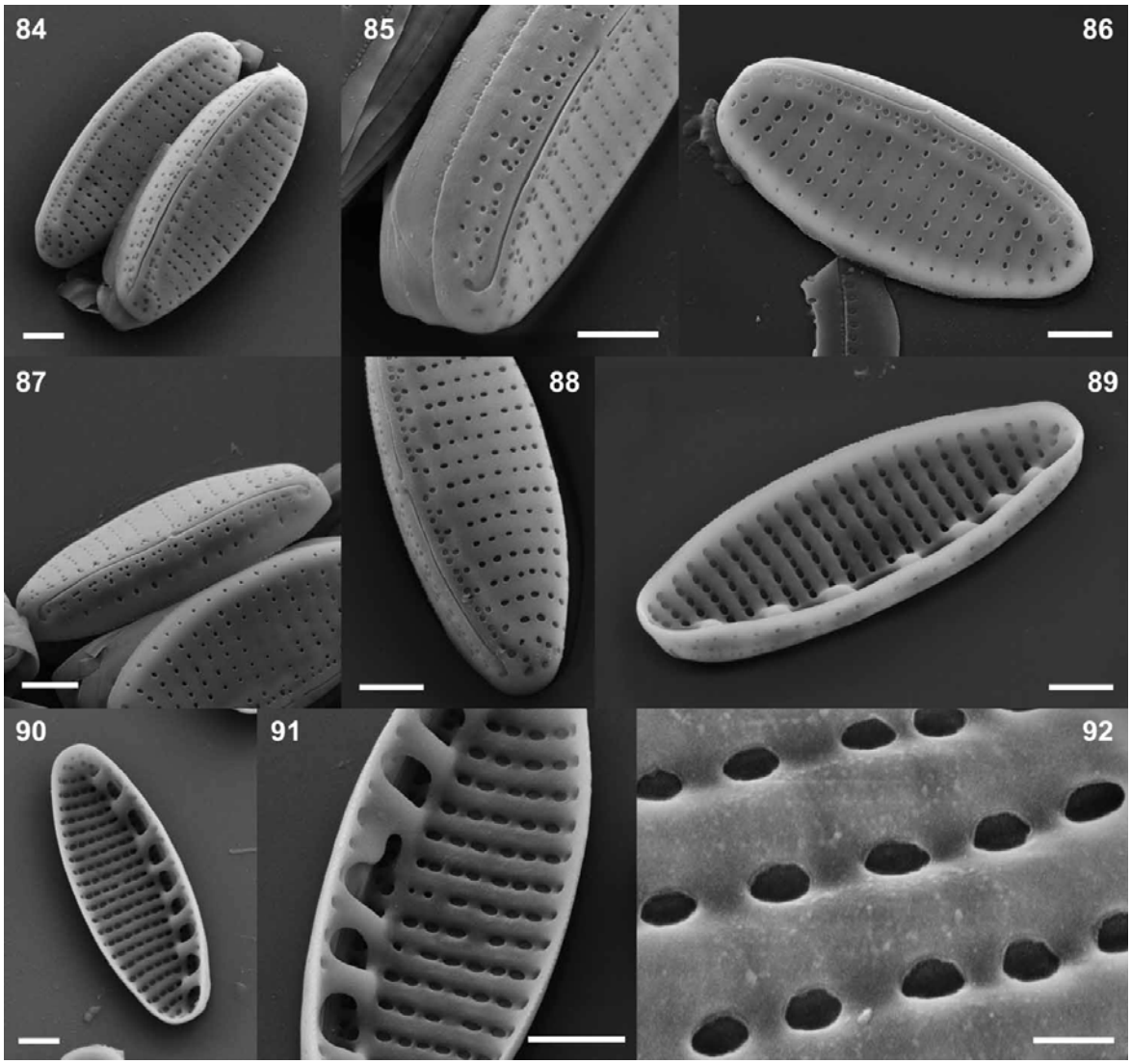
Light microscopy of modern samples from the UK. The valve outline and morphometrics of specimens from UK



Figs 80–83. *Nitzschia boliviana*: type material (Bolivia, South America, ANSP GC 26804), external (Figs 80–81) and internal (Figs 82–83) views. **Figs 80–81**. Valves: note straight or slightly bent central raphe endings; clearly visible hymenes in the raphe canal areolae and in the stria areolae; single row of areolae on the valve mantle. **Fig. 82**. Valve with open girdle band with a single row of areolae. **Fig. 83**. Oblique view, showing fibula details and raphe system; central nodule and terminal helictoglossae. Scale bars = 1 μm.

rivers and a lake are given in Table 1. The valve outline varied from linear–lanceolate in long specimens (Figs 29, 51) to elliptical–lanceolate or even elliptical in the smallest valves (Figs 34–35). The apices were usually slightly protracted, but broader than in *N. inconspicua* (compare Figs 29–35 with Figs 18–25). The fibulae were quite regularly distributed along the raphe canal, except at the centre, where the two median ones were further apart than the others (Figs 29, 31–35, 51–52: an exception is Fig. 30), as in most *N. inconspicua* and *N. frustulum* var. *subsalina*, but not as clearly as in *N. invisitata* (Figs 36–44). However, just as in *N. invisitata*, the fibulae appeared like the margin of a page torn from a ring-bound notebook (Figs 29–35, 51–52). The stria areolae could only rarely be resolved in LM (Figs 51–52).

Electron microscopy of modern samples from UK. The valves of *Nitzschia* sp. 1 differed in several respects from those of the taxa described above. As in *N. frustulum* var. *frustulum*, var. *subsalina* and *N. inconspicua*, the striae of the valve face were uniseriate (Figs 84, 86, 88–91), but with smaller areolae (relative to the interstriae) and within the raphe canal each stria was represented by a triplet of even smaller areolae (Figs 84–86, 88), rather than the single areola of the other taxa. A triplet of small areolae was also present within the raphe canal on the mantle (Figs 85, 87); in addition to the triplets, the mantle had a single row of small round areolae like those of the valve face (Figs 84–85, 87). Externally, some valve face areolae often appeared smaller or occluded along the centre of the valve face (Figs 84, 86). At the boundary between the raphe canal and the valve face the areolae were more widely spaced, creating a narrow plain longitudinal area (Figs 86, 88). Hymenes could be detected internally and bore very fine (ca. 5 nm in diameter) areolae in a hexagonal array (quincunx) (Fig. 92 and Supplementary plate 4); externally, the hymenes were more difficult to detect, except in the areolae of the raphe canal. The raphe was interrupted centrally by a small central nodule (Fig. 91). The external central raphe endings were slightly expanded and either straight (Figs 84–85) or slightly deflected towards the mantle (Figs 86–88). The terminal fissures were very short and either almost straight (Fig. 94) or curved towards the mantle (Figs 85, 87–88), where they often seemed to fuse with an areola (Fig. 85). Among more than nine valves photographed in SEM, none had terminal fissures curved towards the valve face. Hence, although it is impossible to say that valve face curvature never occurs, it was concluded with



Figs 84–92. *Nitzschia* sp. 1: cultured material (clones NIT1008KEL and NIT1009KEL), external (Figs 84–88) and internal (Figs 89–92) views. **Fig. 84**. Disrupted frustule: note the straight central raphe endings. **Fig. 85**. Oblique view showing the structure of the raphe mantle and curved terminal fissure. **Fig. 86**. Whole valve showing a clear strip between the raphe canal triplets of areolae and the valve face striae. **Fig. 87**. Oblique view showing the raphe and mantle. **Fig. 88**. Part of valve with central raphe endings turned towards the mantle. **Figs 89–90**. Valves showing fibulae. **Fig. 91**. Detail of fibulae and central nodule. **Fig. 92**. Detail of areolae, showing their tendency to have hexagonal outlines. In the original photograph, the hymen pores could be seen to be arranged in quincunx (Supplementary plate 4). Scale bars = 1μm or (Fig. 92 only) 200 nm.

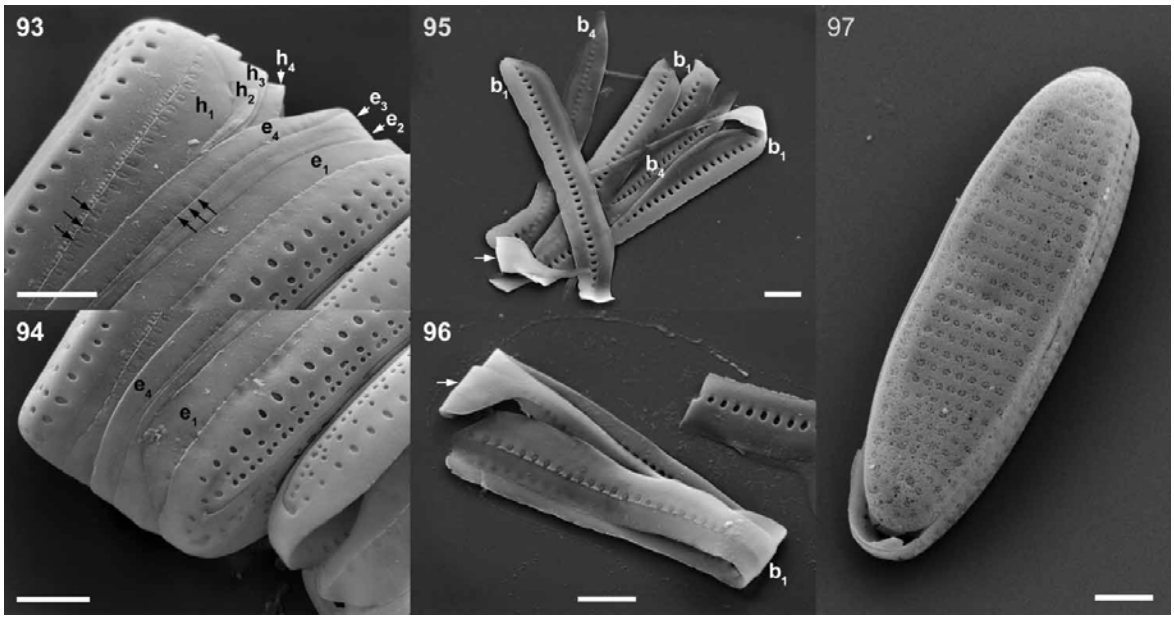
confidence that *N. soratensis* does not have the 1:1 ratio of mantle to valve face curvature expected in *Nitzschia* species (see Mann 1984).

The mature cingulum contained four open bands, of which the first (valvocopula) and fourth bore a single line of round areolae (more closely spaced on the first band) (Figs 93–96). Bands 1 and 4 were also wider than the non-porous bands 2 and 3 (Figs 93–94).

Nitzschia soratensis *E. Morales & Vis 2007 (Figs 26–28, 98–105 supplementary plate 3)*

Original description. Morales & Vis 2007, p. 128, figs 253–256, 277–280.

Light microscopy of type material from Bolivia. Selected valves from the type material of *N. soratensis* are shown in Figs 26–28. Valve shape and metrics were entirely



Figs 93–97. *Nitzschia* sp. 1: cultured material (clones NIT1008KEL and NIT1009KEL), external views. **Figs 93–94**. Opposite ends of a frustule, showing the hypocingulum (h_1–h_4) and epicingulum (e_1–e_4). Note that the bands are open (e.g., e_1 in Fig. 94, h_1, e_2 and e_4 in Fig. 93), and that the first and fourth bands (e_1, h_1, e_4) each bear a single row of areolae, which are more densely spaced on the first than the fourth (e.g., Fig. 93, black arrows on h_1 and e_4). **Fig. 95**. Girdle bands derived from a single frustule. Note that the pore density on some bands (band 4: b_4) is higher than those on others (band 1: b_1). **Fig. 96**. Separated open girdle bands: b_1 is the first band (valvocopula). Note the wide ligula of band 2 (arrow), which closes the opening between the ends of band 1. **Fig. 97**. *Nitzschia abbreviata*: type material, whole valve, external view (for further details, see Trobajo et al. 2012). Scale bars = 1 μm.

within the ranges exhibited by *Nitzschia* sp. 1 (Figs 29–35, Table 1).

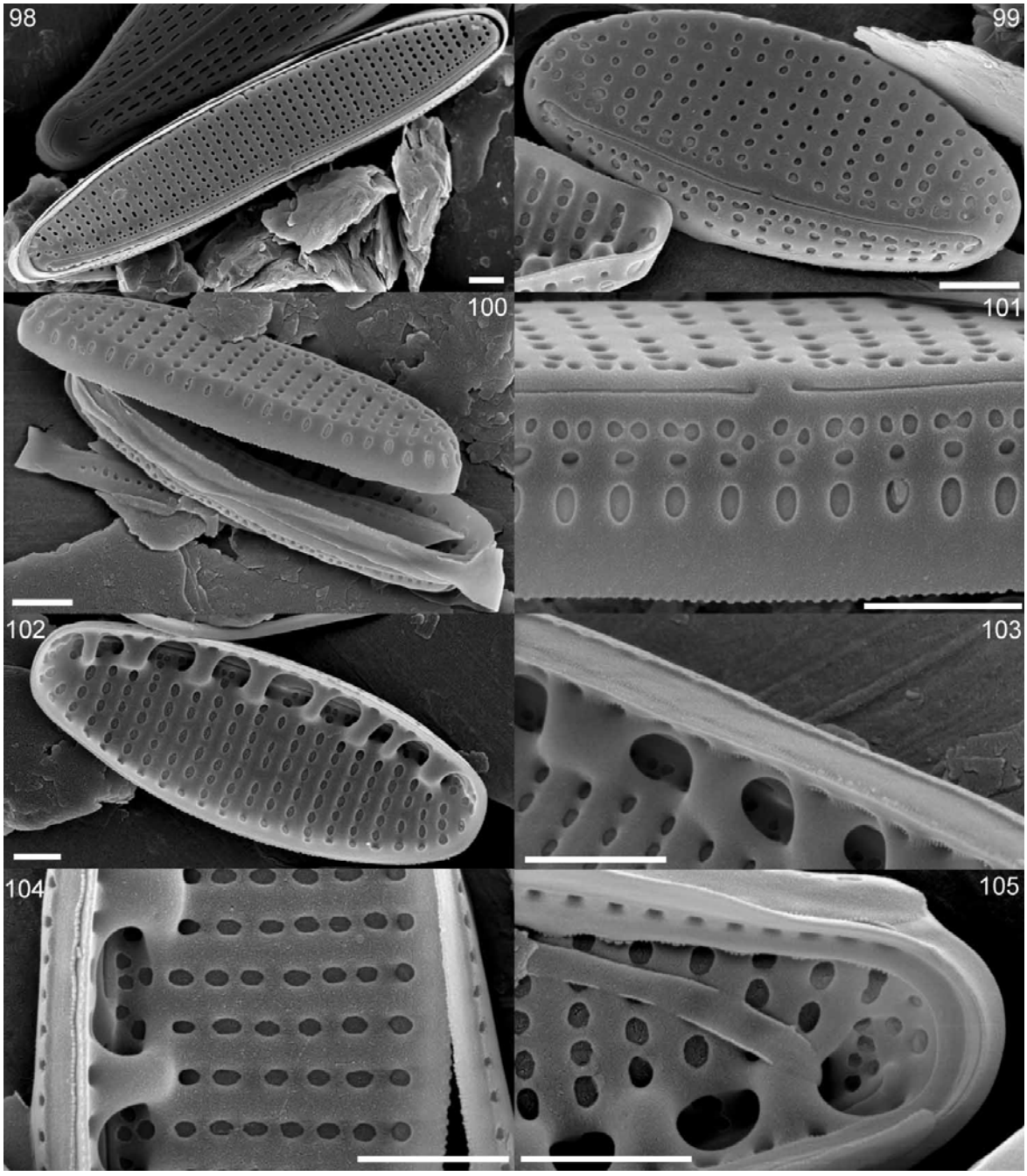
Electron microscopy of type material from Bolivia. Externally, the valves of *N. soratensis* presented uniseriate striae (Figs 98–99) formed by small areolae occluded by hymenes, which were visible, both externally (Figs 99, 101) and internally (Figs 102–105). Striae from the valve face and valve mantle ended in two smaller areolae located adjacent to the raphe, forming a triplet of small areolae within the raphe canal (Figs 98–101). On the opposite side to the raphe canal, there was a single row of elongated areolae located along the valve mantle (Fig. 100). On the mantle adjacent to the raphe, in addition to the triplets, there was a single row of small round or elliptical areolae like those of the valve face (Fig. 101). The raphe was interrupted at the centre and ran along the junction between valve face and valve mantle (Figs 99, 101). The central raphe endings were not bent but were slightly expanded (Fig. 101), whereas the terminal raphe ends curved towards the mantle (Fig. 99). Internally, the fibulae were slightly more widely separated at the centre (Figs 102, 104) and a small central nodule was visible interrupting the raphe (Fig. 104), as in *Nitzschia* sp. 1 (Fig. 91) and the other species studied here. The triplets of areolae were visible internally within the raphe canal (Fig. 104).

Note: according to Morales & Vis (2007, p. 128), the girdle bands are closed, but open ends were clearly visible in the type material examined (Figs 100, 105). The valvocopula had one row of wide areolae (Fig. 105) and a finely fimbriate margin on the pars interior (Fig. 103). Morales & Vis (2007, fig. 280) showed a frustule in girdle view, in which the theca can be seen to comprise four bands, of which the first (the valvocopula, uppermost in their photograph) and the fourth, but not bands 2 and 3, bear a single row of areolae, as in *Nitzschia* sp. 1 (Figs 93–96); moreover, again as in *Nitzschia* sp. 1, the pore density in the valvocopula is less than in band 4.

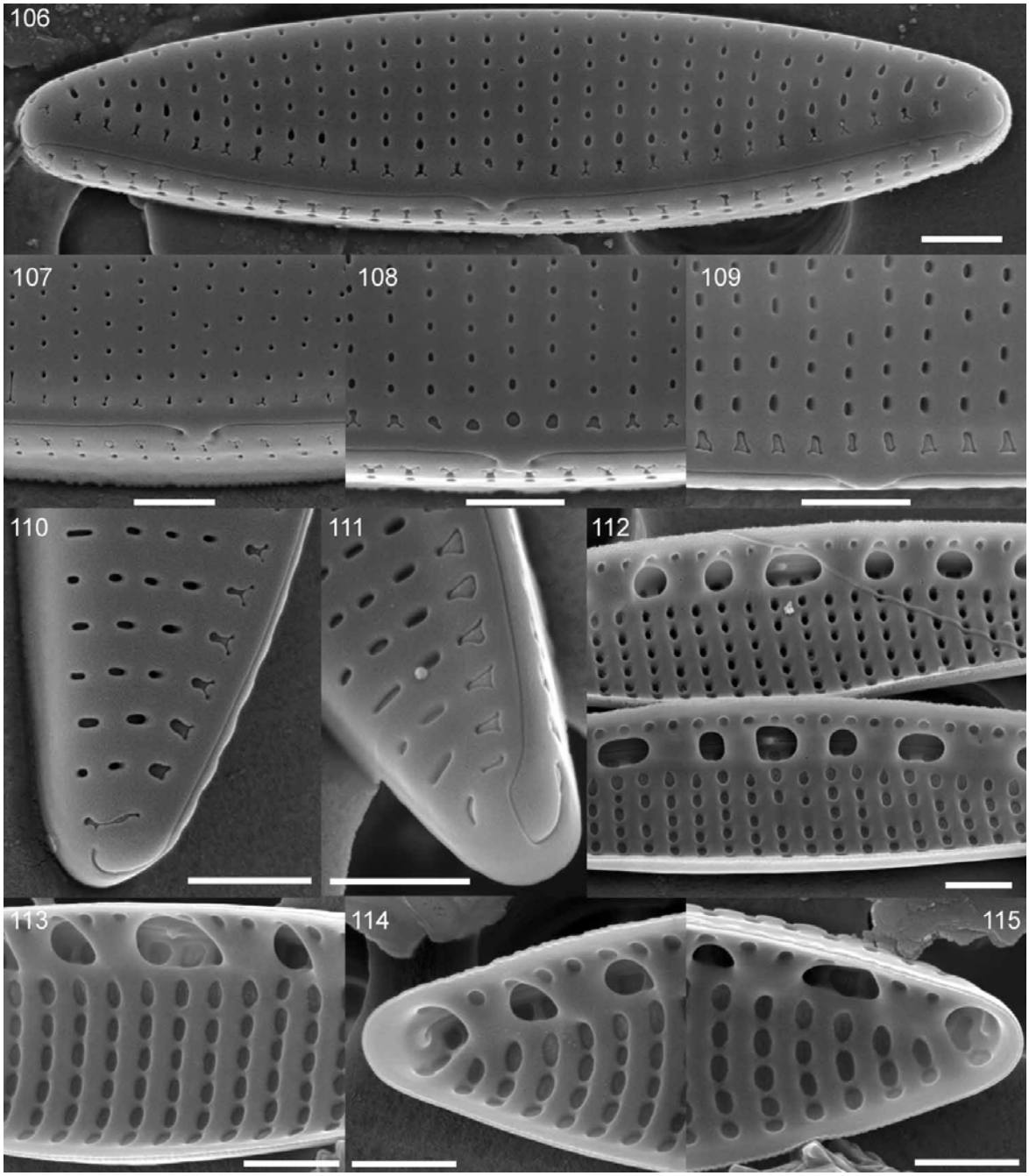
Nitzschia invisitata *Hustedt 1942 (Figs 36–44, 53–54, 106–115)*

An isolectotype slide was examined. Apparently, the Lake Mahalona sample is the only material from which Hustedt recorded *N. invisitata* and he regarded the species as a local endemic of this lake in east-central Sulawesi, Malay Archipelago (Hustedt 1942, p. 235). Hustedt (1942, p. 134) indicated that *N. invisitata* was 'ziemlich häufig' (quite often) in the sample and specimens corresponding to his drawings (Hustedt 1942, figs 297–300) and the type catalogue (Simonsen 1987, pl. 431, figs 1–6) were easily identified.

Light microscopy of type material. Valve outline and morphometric data for *N. invisitata* specimens in the type material are shown in Table 1. Valve outline varied from



Figs 98–105. *Nitzschia soratensis*: type material (Bolivia, South America, ANSP GC 26804), external (Figs 98–101) and internal (Figs 102–105) views. **Figs 98–99**. Large and small valves. Note the clearly visible hymenes in the raphe canal (triplets of areolae) and also in the striae. **Fig. 100**. Oblique view of the valve mantle opposite the raphe canal showing elongated areolae on the mantle; below are open girdle bands, some with one row of areolae. **Fig. 101**. Detail of centre showing one row of areolae below the triplets of areolae on the valve mantle. Raphe interrupted at the central nodule with straight proximal raphe endings. **Fig. 102**. Whole valve. **Figs 103–104**. Detail of central area. Note the delicate fimbriate margin of the valvocopula which underlaps the valve mantle. **Fig. 105**. Detail of valve pole showing girdle bands, at least one of which is open, and the helictoglossa. Scale bars = 1 μm.



Figs 106–115. *Nitzschia invisitata*: type material (Sulawesi, Malay Archipelago. Hustedt Collection, material AS1322), external (Figs 106–111) and internal (Figs 112–115) views. **Fig. 106**. Whole valve. **Figs 107–109**. Details of external central area. Note the variability of the areolae (size and morphology); hymenes clearly visible in the raphe canal areolae of all three valves and in the stria areolae; and proximal raphe endings clearly deflected toward the mantle. **Figs 110–111**. Hooked terminal fissures turned either towards the valve face (Fig. 110) or towards the mantle (Fig. 111). Note the morphological plasticity of the areolae located in the raphe canal, both here and in Figs 107–109. **Figs 112–115**. Parts of valves showing fibulae, central nodule and terminal helictoglossae. Scale bars = 1 μm.

linear–lanceolate in long individuals (Figs 37–38) to more lanceolate as the length reduced (Figs 43–44, 54). As with *N. frustulum* var. *subsalina*, no extremely short valves (<8 μm) were found. All the specimens had very slightly protracted apices. The fibulae were quite regularly distributed along the raphe canal, except at the centre, where the two median ones were further apart than the others (Figs 37–44, 53–54), as in *N. inconspicua* and *N. frustulum* var. *frustulum* and var. *subsalina*. Compared with these taxa, however, the central interspace in *N. invisitata* was usually more clearly differentiated and the fibulae were not small rectangular dots but larger blocks, which expanded towards the valve face. Their overall visual effect of the fibulae was like the margin of a page that has been torn from a ring-bound notebook (Figs 37–44). The stria areolae were easily resolved in LM (Figs 53–54).

While scanning the whole of slide M1/69, a few valves were encountered that were interpreted as initial valves or valves formed by the immediate descendants of initial cells (e.g., Fig. 36). These were ca. 24 μm long and lanceolate, sometimes with a slight central expansion.

Electron microscopy of type material. The striae of the valve face were uniseriate (Figs 106–115), but with rather small areolae externally (Figs 106–111). Within the raphe canal each stria was represented by a single, usually triangular or triradiate areola (Figs 106–111). However, the raphe canal areolae were highly polymorphic and in the central portion of the valve, they tended to be rounded or elliptical (Figs 107–109). The same variability was also observed in the raphe canal areolae located on the mantle (Figs 106, 108). The mantle had two rows of areolae: one near the raphe canal composed of the polymorphic areolae already described and a second located below it, composed of single small rounded areolae (Figs 107–108), similar to those on the valve face. Hymenes could be detected internally and externally; those occluding the areolae of the raphe canal were often more obvious externally than those of the valve face areolae (Figs 109, 111). The raphe was interrupted centrally and the external endings were strongly expanded and deflected towards the mantle (Figs 106–109). The terminal fissures were strongly recurved and turned either towards the valve face or towards the mantle (Figs 110–111). Internally the two central fibulae were more separated, and a central nodule was clearly visible (Figs 112–113). At the poles, there were small helictoglossae internally (Figs 114–115), as in the other species studied here and in most *Nitzschia* species.

Comparative morphometrics

ANOVA analysis showed highly significant differences ($p < 0.001$) for the morphological characters studied, when *N. frustulum* var. *frustulum*, *N. frustulum* var. *subsalina*, *N. inconspicua*, *N. invisitata* and *N. soratensis* were considered (Table 2). *Nitzschia frustulum* var. *frustulum* had consistently and considerably wider valves than the rest of the taxa (Table 2, Fig. 116). Comparing stria densities, *N. invisitata* and *N. soratensis* could be easily distinguished, *N. invisitata* having the lowest stria density and *N. soratensis* the highest (Table 2, Fig. 118). With respect to fibula density, *N. invisitata* and *N. soratensis* were similar and lower than in the other taxa (Table 2, Fig. 117).

Table 2. Results of analysis of variance (ANOVA) of morphological characters for *N. frustulum* var. *frustulum* (1), *N. frustulum* var. *subsalina* (2), *N. inconspicua* (3), *N. invisitata* (4) and *N. soratensis* (5), followed by Games–Howell post-hoc tests, with F-statistics and p values.

Morphological characters	ANOVA		
	$F_{12, 1191}$	p	Games–Howell
Width (μm)	2.3	<0.001	$1 \gg 4 = 5 > 3 > 2$
Fibulae in 10 μm	209	<0.001	$1 > 2 = 3 \gg 5 > 4$
Striae in 10 μm	158	<0.001	$5 \gg 1 > 2 = 3 \gg 4$

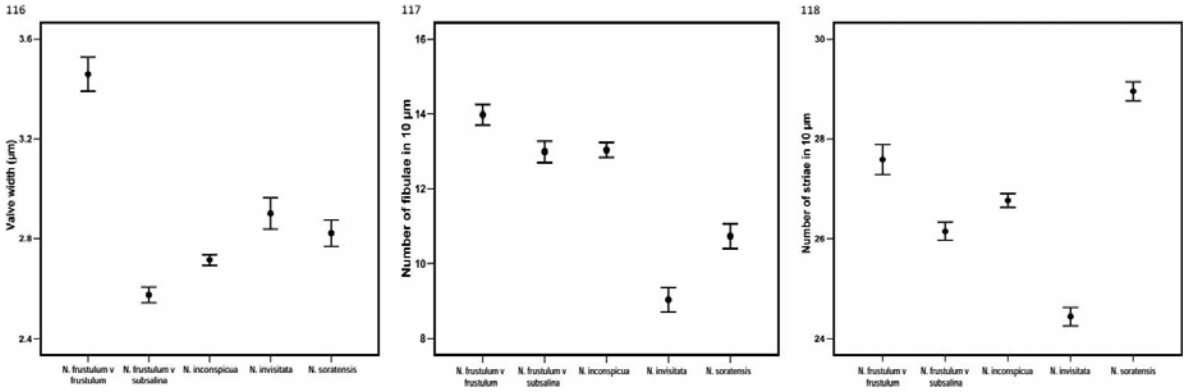
Note: Characters investigated: valve width, number of fibulae and striae in 10 μm. Valve length was not analysed because this characteristic (unlike width, fibula density and stria density) varies greatly during the life cycle and the valves examined cannot be assumed to represent equivalent life-cycle stages.

Discussion

The diatoms studied here are easily confused, especially if only a few valves are found or if insufficient care is taken to relate shape to size. For example, *N. frustulum* and *N. inconspicua* valves sometimes have a similar shape, but this occurs at different lengths (compare Fig. 5 with Figs 13 and 20). Likewise, the poles of longer valves of *N. soratensis* (e.g., Figs 29–30) are roughly as protracted as in some medium-length *N. inconspicua* (Fig. 21), but *N. soratensis* loses this feature in smaller cells, whereas it persists in *N. inconspicua*. Furthermore, long cells (i.e., those formed early in the life cycle) may be perceived as being narrower than they really are, because of the high aspect ratio. It is therefore important to establish a secure basis for comparison, which was done visually in Figs 1–44 by aligning valves that have the same length (except Figs 15–17, 26–28). All the species studied here become more rounded as they get smaller (as do most pennate diatoms), so that they 'converge' morphologically. This may help explain why *N. soratensis*, *N. inconspicua* and other morphologically similar taxa are often confused. Taking into account shape and size variation, stria and fibula density, and ultrastructural details, the following four conclusions can be made.

Nitzschia frustulum *var.* frustulum *is distinct from* N. frustulum *var.* subsalina *and* N. inconspicua

Nitzschia frustulum var. *frustulum* has significantly wider valves than *N. frustulum* var. *subsalina* or *N. inconspicua*,



Figs 116–118. Error bars (mean ± 2 standard error) showing variation of valve width, fibula and stria density in *N. frustulum* var. *frustulum* (type material), *N. frustulum* var. s*ubsalina* (type material), *N. inconspicua* (type material + modern populations), *N. invisitata* (type material) and *N. soratensis* (type material + modern population = as *Nitzschia* sp. 1).

the latter being represented by both type material and modern populations from Spain and the UK in this study. Valve width has been proved to be one of the very few reliable taxonomic characters in species of *Nitzschia* section Lanceolatae, as shown by its low variation when different modern populations are considered, and by its low intraclonal variation, both when subjected to different conditions (e.g., salinity) and during the entire life cycle (Geissler 1970a, b, Wendker & Geissler 1988, Trobajo et al. 2004a, 2006, 2009, 2011, Hlúbiková et al. 2009). Valve width is also known to be valuable in other pennate diatoms from the classic studies of the life cycle by Geitler (1932), although it is not immune to environmental influence (Jahn 1986). Hence the uses of valve width to separate *N. frustulum* var. *frustulum* from *N. frustulum* var. *subsalina* or *N. inconspicua* constitutes a prima facie case for taxonomic separation, even though all three taxa have similar fibula and stria densities and share more-or-less identical ultrastructure.

A further difference supporting separation is that it is likely that *N. frustulum* var. *frustulum* produces much larger initial valves than var. *subsalina* or *N. inconspicua.* Our cultures of *N. inconspicua* produce initial cells no more than 30.1 µm long (personal observations), whereas the type material of var. *frustulum*, in which no initial cells were found, nevertheless contains valves of up to 34 µm. Krammer & Lange-Bertalot (1988) recorded a maximum length for *N. frustulum sensu lato* of 60 µm, but valves having this length in the type or modern populations were not observed. Therefore, our results disagree with both (1) the conclusion of Lange-Bertalot & Simonsen (1978) that *N. frustulum* var. *subsalina* and *N. inconspicua* are both synonymous with *N. frustulum* var. *frustulum*, and (2) a later classification uniting *N. frustulum* var. *subsalina* but not *N. inconspicua* with *N. frustulum* var. *frustulum* (Krammer & Lange-Bertalot 1988, p. 94).

In contrast, no secure basis for separating *N. inconspicua* from *N. frustulum* var. *subsalina* was found. There are only minute differences between these taxa in valve dimensions and none in fibula and stria densities, and there are also no ultrastructural differences. Both taxa can live in saline habitats (Hustedt 1925, Trobajo et al. 2011, as *Nitzschia frustulum sensu lato*). Therefore the results agree with Lange-Bertalot & Simonsen (1978, p. 27) and Lange-Bertalot (1993, p. 142) that *N. inconspicua* and *N. frustulum* var. *subsalina* should be treated as synonyms.

Nitzschia inconspicua *should be recognized as a separate species, of which* N. frustulum *var.* subsalina *and* N. boliviana *are synonyms*

In 1993, Lange-Bertalot modified the classification in Lange-Bertalot & Simonsen (1978) and Krammer & Lange-Bertalot (1988), arguing that *N. inconspicua* did not deserve recognition as a separate species but might perhaps be worth recognition as a variety. Lange-Bertalot (1993) argued that *N. frustulum* and *N. inconspicua* are only phenotypically, not genotypically differentiated, citing Wendker's (1990a) studies of the Schlei Estuary (in which *N. frustulum sensu lato* was abundant) in support. The recent benthic diatom flora for central Europe by Hofmann et al. (2011) adopted Lange-Bertalot's (1993) classification, separating *N. frustulum* var. *frustulum* and var. *inconspicua* within *N. frustulum*. In the work cited by Lange-Bertalot, Wendker (1990a, p. 115) stated that she treated *N. frustulum* and *N. inconspicua* as synonyms ('in der vorliegenden Arbeit als Synonym zu *N. frustulum* aufgefaßt'). She did not explain this conclusion, but in a companion paper, Wendker (1990b) analysed variation in and among Schlei populations of *N. frustulum sensu lato* and suggested that the smaller, narrower and more rounded '*inconspicua*' morphology, occurring in the lower salinity reaches of the Schlei, intergraded into the longer, wider and more acute-ended '*frustulum*' morphology, occurring nearer the sea. However, she presented no direct evidence that the variation

was phenotypic and deferred a decision on the conspecificity of *N. frustulum* and *N. inconspicua* until further studies could be made of clonal cultures ('Weiterführende Untersuchungen an Klonkulturen sollten als Fragestellung die eventuelle Konspezifität dieser beiden Taxa berücksichtigen'). Our studies of *N. inconspicua* cultures (personal observations) show that they never produce the wider valves characteristic of *N. frustulum sensu stricto*, nor such long valves as occur in the type material of *N. frustulum*.

Two comments can be made about the classification of *N. frustulum* and *N. inconspicua* as varieties of a single species. First, as noted already by Lange-Bertalot (1993), the correct name for *N. inconspicua*, if it is regarded as synonymous with var. *subsalina* and treated as a variety of *N. frustulum*, is var. *subsalina*. The name s*ubsalina* has priority (dating from 1925, in the original description by Hustedt) because Grunow never made a valid new combination of var. *inconspicua*. Hence the recent treatment by Hofmann et al. (2011) would need modification: (1) the diatom described there as *N. frustulum* var. *inconspicua* (p. 446 and pl. 112, figs 35–40) should have been called *N. frustulum* var. *subsalina*; and (2) most of the specimens presented there as *N. abbreviata* (p. 446 and pl. 112, figs 21–27) most likely correspond to *N. soratensis* (for the explanation see below).

Second, treating *N. inconspicua* as a variety of *N. frustulum* means that it is less likely to be recorded (some diatomists do not use taxonomic ranks lower than species), especially if it is believed that it is only a phenotypic modification (the interpretation of Lange-Bertalot 1993) or a life history stage (the interpretation of Hofmann et al. 2011, p. 446), which our morphometric data (e.g., on the length range: as stated in previous section) indicate it is not. As a result, the ecology and biogeographical distribution of these two taxa (*N. frustulum* and *N. inconspicua*) will always be confused.

Nitzschia inconspicua should be treated as an independent species. In LM, distinguishing *N. inconspicua* from *N. frustulum* is not impractical, because of the latter's wider valves. The more difficult (although not impossible) separation in LM is between *N. inconspicua* (fully or nearly fully euryhaline, from our own experimental data on clones and salinity) and the more rounded diatom *Nitzschia* sp. 1 (which is a strictly freshwater species, also from our own experimental data). *Nitzschia* sp. 1 as stated below should be called *N. soratensis* (cf. Krammer & Lange-Bertalot 1988, pl. 69, figs 1–13, where both *N. inconspicua* and *N. soratensis* are illustrated together as *N. inconspicua*).

The measurements made on type material of *N. boliviana* agreed well with those given by Morales & Vis (2007), who gave 6.0–9.6 × 2.4–2.8 μm, with 27.5–30 striae and 12.5–15 fibulae in 10 μm, except for the stria densities; for these, our figures were lower than given by Morales & Vis (27.5–30 striae in 10 μm), ours corresponding to those of *N. inconspicua* and *N. frustulum* var. *subsalina*, rather than to the higher densities of *N. soratensis* and *Nitzschia* sp. 1 (Table 1). *Nitzschia boliviana* therefore seems to be conspecific with *N. inconspicua* and should be considered synonymous because their ultrastructural features are very similar in all aspects, and full overlap between the two species in metric characters (Table 1).

Nitzschia inconspicua, N. abbreviata *and* N. invisitata *are not synonyms*

Lange-Bertalot & Krammer (1987, p. 3) and Krammer & Lange-Bertalot (1988, p. 95) suggested that *N. abbreviata* and *N. inconspicua* might be conspecific, both having a simple elliptical outline and similar valve dimensions and striation density. Hofmann et al. (2011), by contrast, separated *N. inconspicua* and *N. abbreviata*. Our examination of *N. abbreviata* type material from Lake Tanganyika (western branch of the African Rift) (Trobajo et al. 2012) revealed several small diatom species, all similar in LM but with different ultrastructures. The ultrastructure most likely to correspond to the *N. abbreviata* lectotype (Fig. 97: for other images, see Trobajo et al. 2012) differs from *N. inconspicua* and *N. frustulum* in having valve areolae occluded externally by coarse cribra and striae that become biseriate in the raphe canal. In addition, the striation density of valves with this type of ultrastructure, assigned to *N. abbreviata* (Trobajo et al. 2012), is higher (generally 27–34 in 10 μm) than in *N. inconspicua* and *N. frustulum*, and the areolae cannot be resolved in LM. Therefore, although *N. abbreviata* and *N. inconspicua* are very similar in LM, as noted by Hofmann et al. (2011, p. 431), these two are separate from each other and from *N. frustulum*. Besides *N. abbreviata* itself, the Lake Tanganyika material contains a species that shares the same ultrastructure as *N. inconspicua* ('ultrastructure 3' in Trobajo et al. 2012) and has similar dimensions and pattern densities as *N. inconspicua*, raising the possibility that *N. abbreviata* and *N. inconspicua* may coexist in some localities. However, until now, no observation of *N. abbreviata* with SEM examination could certify the presence of this species in Europe.

Our data also support *N. invisitata* as an independent species, which has been confirmed, so far, only from the type locality in Sulawesi. The species is clearly differentiated in LM from the other species treated here, and also from *N. abbreviata*, by the coarser areolae in the striae (well illustrated also by Simonsen 1987, pl. 431, figs 1–6), relatively low striation density, and larger and more distantly spaced fibulae: the median pair of fibulae are usually very clearly separated, rather more so than in the other species (this feature was noted in the original description and figures: see Hustedt 1942, p. 134). Diatoms identified as *N. invisitata* have been recorded from Kenya by Owen et al. (2004, 2008) and, interestingly, had a much higher alkalinity optimum than *N. frustulum* and *N. inconspicua*, recorded in the same studies. However, no images

of the Kenyan *N. invisitata* were published and none of the original samples remain (R.B. Owen, pers. comm.). It is therefore unclear, especially given the confusion in the taxonomy of these small *Nitzschia* species, whether Kenyan and Sulawesian *N. invisitata* are conspecific.

In its ultrastructure, *N. invisitata* differs from the other species studied here in the central raphe endings, which are not straight but deflected, and in the shape and polymorphism of the areolae in the raphe canal, which are generally triangular or triradiate. With respect to the morphology of the raphe canal pores, *N. invisitata* is similar to *N. kahlii* Lange-Bertalot & Rumrich from South America (central Chile, Rumrich et al. 2000, pl. 165, figs 3, 89), and also to an unnamed species ('ultrastructure 2') found in the type material of *N. abbreviata* from Lake Tanganyika (Trobajo et al. 2012, figs 37–44). *Nitzschia invisitata* is not the same as either of these, however, since *N. kahlii* lacks central raphe endings (Rumrich et al. 2000), while in the Tanganyikan diatom, the valve face areolae are subdivided by transapical bars, the central raphe endings are straight, and there are no terminal fissures (Trobajo et al. 2012).

Nitzschia soratensis ***is the correct name for a small, blunt-ended, strictly freshwater species previously identified in Europe as*** **N. inconspicua** ***or*** **N. abbreviata**

A major impetus for our study was the realization that, within European samples, there appear to be two very similar *Nitzschia* species, one with slightly more rounded apices and finer striation than the other. Both were illustrated as '*N. inconspicua*' by Lange-Bertalot (1977, figs 22–25, 27a) and Krammer & Lange-Bertalot (1988, pl. 69, figs 1–10), but the more rounded one, which is described here in detail from UK material, is clearly differentiated by its ultrastructure (i.e., *Nitzschia* sp. 1 having triplets of small areolae within the raphe canal while *N. inconspicua* has uniseriate stria within the raphe canal, Figs 84–88 vs. Figs 70–74) from the others and in having, on the whole, higher stria density and lower fibula density than *N. inconspicua* (see Table 1 and Figs 117–118). Also, the fibulae of *Nitzschia* sp. 1 have a different appearance in LM, resembling the margin of a page torn from a ring-bound notebook while *N. inconspicua* resembled *Nitzschia* sp. 1 (compare Figs 18–25 vs. Figs 29–35).

Nitzschia sp. 1 was wrongly called *N. abbreviata* by Hofmann et al. (2011, p. 431, figs 21–27), which included some of the same images that were used to illustrate '*N. inconspicua*' by Krammer & Lange-Bertalot (1988). As shown by our examination of type material (Fig. 97, and see Trobajo et al. 2012), *N. abbreviata* differs from *Nitzschia* sp. 1 by having: (1) complex cribra in relatively large areolae (relative to the width of the interstriae), whereas only a hymen is present in *Nitzschia* sp. 1; (2) deeper and more clearly differentiated mantles; (3) elongate rather than more or less circular areolae on the mantle (on both sides of the valve, both adjacent to and opposite the raphe); and (4) terminal fissures that can bend towards either the valve face or mantle, rather than only towards the mantle. Also, in *N. abbreviata*, the stria structure in the raphe canal is variable: sometimes there are double or triple areolae, which tend to merge with each other, but more often there are single areolae. In *Nitzschia* sp. 1, however, there are almost always triplets of small areolae in the raphe canal, which are separated from the valve face by a narrow plain strip. Finally, there are differences in girdle structure between *N. abbreviata* and *Nitzschia* sp. 1, the most abvalvar band of *N. abbreviata* bearing short striae containing three areolae apiece, whereas the equivalent band in the rounded UK form has a simple longitudinal line of elliptical areolae.

Nitzschia sp. 1 seems to be conspecific with *N. soratensis* described by Morales & Vis (2007). The ultrastructure of the raphe canal is the same, with neat triplets of areolae separated from the valve face stria by a plain strip; there is a discrete row of elliptical areolae on the mantle (at least on the side opposite the raphe: Morales & Vis 2007, fig. 280); and the length, width, fibula density and stria density are similar. The European *Nitzschia* sp. 1 and the Bolivian *N. soratensis* cannot be separated. *Nitzschia soratensis* has also been illustrated in SEM from a sample from the Chilean Altiplano and misidentified as *N. acidoclinata* Lange-Bertalot by Rumrich et al. (2000, pl. 167, fig. 3).

Therefore, the illustrations of some *N. inconspicua* in Krammer & Lange-Bertalot (1988, pl. 69, figs 6–10) and those of '*N. abbreviata*' in Hofmann et al. (2011, pl. 112, figs 21–27) represent *N. soratensis*. *Nitzschia soratensis* appears to be a strictly freshwater species (already at a salinity of 3 ppt, the growth of our clones of *N. soratensis* was almost completely suppressed and cells were unable to move and separate, thus forming chains), whereas the diatom that is most often confused with it, *N. inconspicua*, extends from freshwater into brackish and marine waters (Chu et al. 1996, Trobajo et al. 2011, Rovira et al. 2012b, and our unpublished data on cultures and salinity tolerances). Lange-Bertalot (1993) characterized what we now call *N. soratensis* as a diatom occurring 'im Süsswasser mit nur mittlerem Elektrolytgehalt' and Hofmann et al. (2011) noted that confusion of *N. inconspicua* with *N. soratensis* (= their *N. abbreviata*) has led to calculation of over-high halobion indices. Currently, experiments are being conduted to determine the preferences and tolerances of *N. soratensis* clones to some environmental variables, for comparison with *N. inconspicua* [for which, see (1) Trobajo et al. (2004a) where *N. inconspicua* and *N. frustulum* were considered as conspecific taxa; and (2) Trobajo et al. (2011) where *N. inconspicua* was misidentified as *N. frustulum*]. Morales & Vis (2007) stated that *N. soratensis* 'prefers slightly eutrophic waters (210 μS cm^{-1}) with alkaline pH (8.6) and cold water temperature (17 °C)'; other records (Morales et al. 2007, with environmental data from

Table 3. LM morphological comparisons differentiating the four reassigned *Nitzschia* species, together with ecological preferences.

Species comparison	Apex shape	Valve width	Stria	Fibula	Ecology
N. frustulum versus *N. inconspicua*	**	Determinant feature: wider valves for *N. frustulum*, always >3 μm	**	**	Not known*
N. frustulum versus *N. soratensis*	Slightly protracted, narrow in *N. frustulum*; slightly protracted, broad in *N. soratensis*	Wider valves for *N. frustulum*, always >3 μm	Generally less conspicuous in *N. soratensis*	Lower fibula density in *N. soratensis* Shape: small rectangular in *N. frustulum*; 'torn notebook' in *N. soratensis*	Not known*
N. frustulum versus *N. invisitata*	**	Wider valves for *N. frustulum*, always >3 μm	Generally lower density in *N. invisitata*	Shape: small rectangular in *N. frustulum*; 'torn notebook' in *N. invisitata*	Not known*
N. inconspicua versus *N. soratensis*	Slightly protracted, narrow in *N. inconspicua*; slightly protracted, broad in *N. soratensis*	**	Generally less conspicuous in *N. soratensis*	Shape: small rectangular in *N. inconspicua*; 'torn notebook' in *N. soratensis*	*N. inconspicua*: brackish–marine/euryhaline; *N. soratensis*: strictly freshwater
N. inconspicua versus *N. invisitata*	**	**	Slightly lower density in *N. invisitata*	Shape: small rectangular in *N. inconspicua*; 'torn notebook' in *N. invisitata*	Not known*
N. soratensis versus *N. invisitata*	Slightly protracted, broad in *N. soratensis*; slightly protracted, narrow in *N. invisitata*	**	Lower density in *N. invisitata*	**	Not known*

Note: *, comparison not possible because the two taxa have previously been confused or the ecological preference is known for only one of the pair; **, the two taxa cannot be distinguished by this character.

McClintic et al. 2003) give a range of conductivities, pH and temperatures of 110–210 μS cm^{-1}, 7.4–8.6 and 10–17 °C, respectively.

In order to make advances in knowing the ecology of *N. inconspicua* and *N. soratensis*, it is obviously essential to be able to identify them reliably. In SEM and TEM, there is no possibility of confusion, but in ecological studies and biomonitoring it is impractical to use SEM routinely, and LM diagnosis is therefore essential. The ranges of length, width, stria density and fibula density overlap between *N. inconspicua* and *N. soratensis* and the two species do sometimes occur together in the freshwater habitats (though never with both at high abundances at the same time: our unpublished data). However, a combination of characters allows most valves of these two species to be identified with confidence in LM, by (in each case, *N. soratensis* is given first):

- more rounded vs. somewhat acute poles (compare Figs 26–35 with Figs 10–25);
- fibulae like the margin of a page torn from a ring-bound notebook (and generally more widely spaced) vs. small rectangular dots;
- striae less easy to resolve (fainter striae, due to smaller areolae), with invisible areolae (ca. 30 in 10 μm) vs. striae more easy to resolve with the possible observation of areolae (ca. 17 in 10 μm) and larger than in *N. soratensis*.

The LM differences between all four reassigned *Nitzschia* species considered in this work are summarized in Table 3.

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Supplementary material

The following supplementary material, comprising 4 supplementary plates, is available for this article accessible via the Supplementary Content tab on the article's online page.

Supplementary Material - Plate 1 *Nitzschia inconspicua*
Figs 1–14. *Nitzschia inconspicua* from modern populations of the Ebro Estuary. Figs 1–10. Light micrographs (bright field optics) illustrating size reduction, scale bar = 5 μm. Figs 11–14. SEM micrographs. Fig. 11. Valve interior showing central nodule and fibula details. Figs. 12. Valve exterior with straight central raphe endings. Fig. 13. Detail of the central raphe endings, note the clearly visible hymenes in the raphe canal poroids and also in the stria poroids. Fig 14. Valve exterior with central raphe endings turned towards the mantle. Scale bars = 1 μm.

Supplementary Material - Plate 2 *Nitzschia boliviana*
Figs 1–22. *Nitzschia boliviana* from type material (Sorata Department, Bolivia, South America. ANSP GC 26804). Figs 1–20. Light micrographs (bright field optics) illustrating size reduction, scale bar = 10 μm. Figs 21–22. SEM micrographs. Fig. 21. General aspect of valve exterior. Fig. 22.Valve interior showing central nodule and fibula details. Scale bars = 1 μm.

Supplementary Material - Plate 3 *Nitzschia soratensis*
Figs 1–24. *Nitzschia soratensis* from type material (Sorata Department, Bolivia, South America. ANSP GC 26804). Figs 1–20. Light micrographs (bright field optics) illustrating size reduction. Figs 5, 9 and 15 illustrating both *Nitzschia soratensis* (a) and *Nitzschia boliviana* (b) co-occurring, scale bar = 10 μm. Figs 21–24. SEM micrographs. Fig. 21. Valve interior showing central nodule and fibula details. Fig. 22. Valve exterior showing pore arrangement on the valve face. Fig. 23. Detail of centre outside, showing the triplets of poroids on the valve mantle and valve face. Raphe interrupted at the central nodule. Fig. 24. Detail of a large specimen (centre) surrounded by accompanying flora, *Reimeria seriata* (W. Gregory) Kociolek & Stoermer (below) and *Navicula* sp. (above). Scale bars Figs 21-23 = 1 μm, Fig. 24 = 10 μm.

Supplementary Material - Plate 4 *Nitzschia soratensis*
Detail of the areolae illustrated in Fig. 92, showing hymen pores arranged in quincunx. Scale bar = 200 nm.

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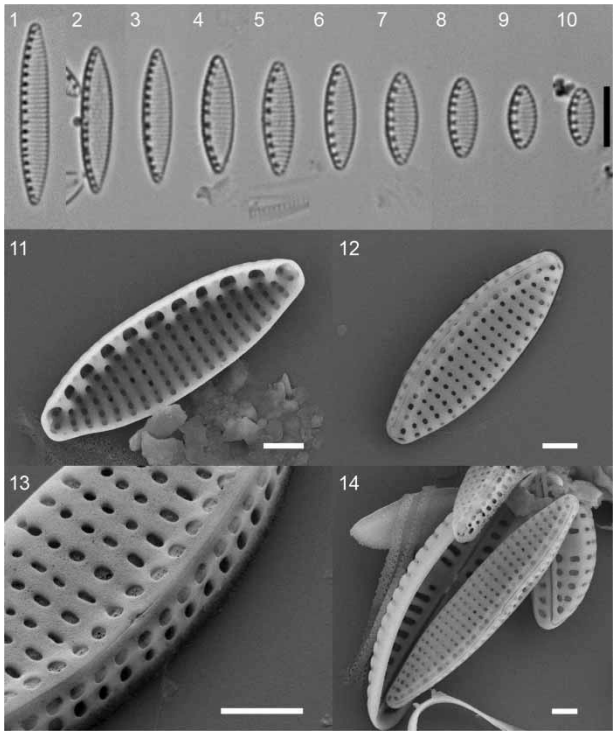
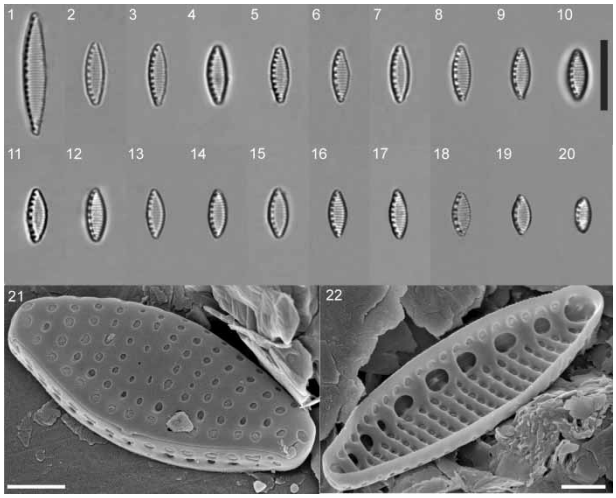
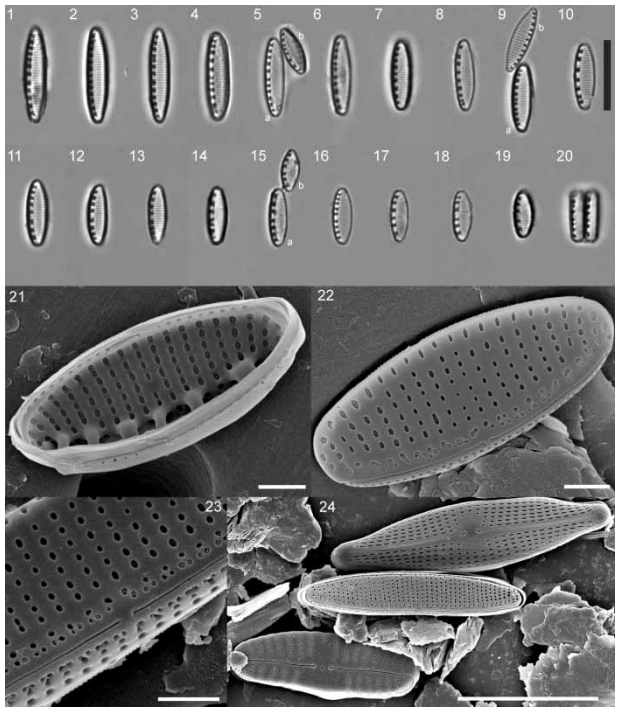


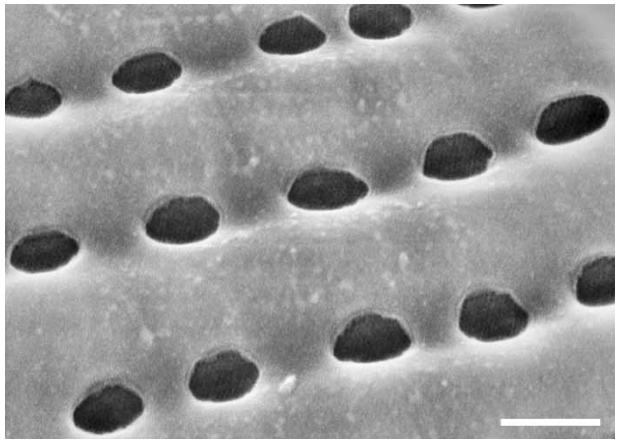
Supplementary Material - Plate 1 *Nitzschia inconspicua*
Figs 1–14. *Nitzschia inconspicua* from modern populations of the Ebro Estuary. Figs 1–10. Light micrographs (bright field optics) illustrating size reduction, scale bar = 5μm. Figs 11–14. SEM micrographs. Fig. 11. Valve interior showing central nodule and fibula details. Figs. 12. Valve exterior with straight central raphe endings. Fig. 13. Detail of the central raphe endings, note the clearly visible hymenes in the raphe canal poroids and also in the stria poroids. Fig 14. Valve exterior with central raphe endings turned towards the mantle. Scale bars = 1 μm.



Supplementary Material - Plate 2 *Nitzschia boliviana*
Figs 1–22. *Nitzschia boliviana* from type material (Sorata Department, Bolivia, South America. ANSP GC 26804). Figs 1–20. Light micrographs (bright field optics) illustrating size reduction, scale bar = 10 μm. Figs 21–22. SEM micrographs. Fig. 21. General aspect of valve exterior. Fig. 22.Valve interior showing central nodule and fibula details. Scale bars = 1 μm.



Supplementary Material - Plate 3 *Nitzschia soratensis*
Figs 1–24. *Nitzschia soratensis* from type material (Sorata Department, Bolivia, South America. ANSP GC 26804). Figs 1–20. Light micrographs (bright field optics) illustrating size reduction. Figs 5, 9 and 15 illustrating both *Nitzschia soratensis* (a) and *Nitzschia boliviana* (b) co-occurring, scale bar = 10 μm. Figs 21–24. SEM micrographs. Fig. 21. Valve interior showing central nodule and fibula details. Fig. 22. Valve exterior showing pore arrangement on the valve face. Fig. 23. Detail of centre outside, showing the triplets of poroids on the valve mantle and valve face. Raphe interrupted at the central nodule. Fig. 24. Detail of a large specimen (centre) surrounded by accompanying flora, *Reimeria seriata* (W. Gregory) Kociolek & Stoermer (below) and *Navicula* sp. (above). Scale bars Figs 21-23 = 1 μm, Fig. 24 = 10 μm.



Supplementary Material - Plate 4 *Nitzschia soratensis*
Detail of the areolae illustrated in Fig. 92, showing hymen pores arranged in quincunx. Scale bar = 200 nm.

Chapter 5

Rovira, L., Trobajo, R., Sato, S., Ibáñez, C. & Mann, D.G. **Genetic and ecophysiological diversity in the diatom *Nitzschia inconspicua*.** Journal of Phycology (under review).

GENETIC AND ECOPHYSIOLOGICAL DIVERSITY IN THE DIATOM *NITZSCHIA INCONSPICUA*[1]

Laia Rovira
Institute for Food and Agricultural Research and Technology (IRTA), Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540, St. Carles de la Ràpita, Catalonia, Spain

Rosa Trobajo
Institute for Food and Agricultural Research and Technology (IRTA), Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540, St. Carles de la Ràpita, Catalonia, Spain

Shinya Sato
Royal Botanic Garden Edinburgh, Edinburgh EH3 5LR, Scotland, UK

Carles Ibáñez
Institute for Food and Agricultural Research and Technology (IRTA), Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540, St. Carles de la Ràpita, Catalonia, Spain

and *David G. Mann*[2]
Royal Botanic Garden Edinburgh, Edinburgh EH3 5LR, Scotland, UK

Running title: Diversity within *N. inconspicua*

[1] Received

[2]Author for correspondence: e-mail d.mann@rbge.ac.uk

ABSTRACT

Nitzschia inconspicua is an ecologically important diatom species, which is believed to have a widespread distribution, to occur in a wide variety of freshwater and brackish ecosystems, and to be tolerant to salinity variation and organic or nutrient pollution. However, its identification is not straightforward, due to the small size of its cells, which have few diagnostic characters. Moreover, there is no information on genetic diversity and ecophysiological variation within the species. We used morphological, molecular (*rbc*L and LSU D1/D3), ecophysiological (salinity preference) and reproductive data to investigate whether *N. inconspicua* constitutes a single species with a broad ecological tolerance or two or more cryptic species with shared or different ecological preferences. Molecular genetic data for strains isolated from upstream and deltaic sites in the Ebro river catchment (Spain) revealed seven *N. inconspicua* genotypes, belonging to three clades. Two of the clades also contained other, long-established and morphologically distinct *Nitzschia* and *Denticula* species, making *N. inconspicua* paraphyletic and in need of taxonomic revision. Most genotypes were observed to be automictic, exhibiting paedogamy, and so the biological species concept cannot be used to help establish species boundaries. Although there were morphological differences among isolates, we found no consistent differences among genotypes belonging to different clades, which are definable thus far only through sequence data. Nevertheless, separating the genotypes could be important for ecological purposes because three different salinity responses occurred among them, underlining the value of combining morphological studies with DNA barcoding in developing future biomonitoring strategies.

KEYWORDS: cryptic species, diatoms, ecophysiology, genetic diversity, LSU rDNA, molecular phylogeny, *Nitzschia, Nitzschia inconspicua*, paraphyletic species, *rbc*L, salinity responses.

INTRODUCTION

Our ability to investigate the ecology, biogeography, evolution, stratigraphy, and paleoecology of diatoms and to use these organisms in biomonitoring and biotechnology is underpinned by our ability to identify species reliably and unequivocally and to link data together via species names. Separation and identification of diatom species mostly relies upon morphological features of the frustules. However, morphology-based identifications are not always straightforward or practical, especially in those species that possess very few characters visible in the LM or show a high degree of morphological variability, which can lead to unreliable morphological diagnoses. Moreover, the results of recent molecular studies have revealed cryptic diversity, suggesting that diatom biodiversity has been greatly underestimated. For example, genetically distinct entities have been found within morphologically circumscribed species in *Chaetoceros, Cyclotella, Cylindrotheca, Eunotia, Hantzschia, Navicula, Nitzschia, Pinnularia, Pseudo-nitzschia, Sellaphora* and *Skeletonema* (e.g. Beszteri et al. 2005, 2007, Créach et al. 2006, Amato et al. 2007, Evans et at. 2007, Vanormelingen et al. 2008, Trobajo et al. 2009, Vanelslander et al. 2009, Kooistra et al. 2010, Poulíčková et al. 2010, Souffreau et al. 2012, Vanormelingen et al. 2013, in press). However, the functional significance of this cryptic diversity is still unclear because studies on the physiology, ecology or geographical distributions of cryptic and pseudocryptic species are still scarce. Exceptions include *Chaetoceros socialis* (Degerlund et al. 2012a, Huseby et al. 2012), *Pinnularia borealis* (Souffreau et al. 2012), the *Sellaphora pupula* complex (Poulíčková et al. 2008) and the *Skeletonema costatum* complex (Gallagher 1982, Kaeriyama et al. 2011).

Nitzschia is one of the largest diatom genera (Mann 1986), notorious for its taxonomic difficulty. *Nitzschia inconspicua* Grunow belongs to section *Lanceolatae*, known as a particularly challenging group because most have small cells and delicate structure, and offer few diagnostic characters (Lange-Bertalot and Simonsen 1978, Kobayasi 1985). *N. inconspicua* is considered to be a widespread diatom found in several types of ecosystems (from freshwater to brackish/marine) and often in high abundances (Fawzi et al. 2002, Ulanova et al. 2009, Rovira et al. 2012a, b). Because of its tolerance to salinity and to organic or nutrient pollution (Krammer and Lange-Bertalot 1988, Van Dam et al. 1994, Gomà et al. 2005, Naymik et al. 2005, Licursi et al. 2010, Delgado et al. 2012), *N. inconspicua* has been included in most diatom-based indices

and suggested as potential bioindicator of anthropogenic stress (e.g. Potapova et al. 2004, Kelly et al. 2008, Rovira et al. 2012b).

However, in spite of being an ecologically important species, the identification of *N. inconspicua* based on morphological characters has not always been clear (Lange-Bertalot 1977, 1993, Lange-Bertalot and Simonsen 1978, Trobajo et al. 2004a, b, Rovira et al. 2012a, b); and it has often been combined with *N. frustulum*, e.g. in studies of Mediterranean shallow coastal lagoons (in Empordà wetlands), where the *N. frustulum–inconspicua* complex was abundant and had value as an ecological indicator (Trobajo et al. 2004b). It is a small to medium sized diatom, whose ranges of size, shape, fibula and stria density mostly overlap with those of other species (such as *N. abbreviata*, *N. costei, N. epiphytica*, *N. frustulum*, *N. innominata*, *N. invisitata* and *N. soratensis*). Nonetheless, recent investigations have clarified the separation of *N. inconspicua* from similar small *Nitzschia* species (Trobajo et al. 2013) and morphological and growth responses to environmental parameters have been documented through experimental studies (Trobajo et al. 2004a, 2011; in both of these *N. inconspicua* was thought to be synonymous with, and referred to as, *N. frustulum*). Most recently, the life cycle of two strains of *N. inconspicua* has been investigated and shown to have paedogamous auxosporulation (Mann et al. 2013). However, these studies have been based on only one or two strains of *N. inconspicua,* giving no information on the genetic diversity and physiological variation (if any) within the species. It is therefore unknown whether *N. inconspicua* constitutes a single species with a broad ecological tolerance or two or more cryptic or pseudocryptic species with shared or different ecological preferences.

Our aim in this work was to use morphological, molecular (*rbc*L and LSU D1/D3), ecophysiological (salinity preferences and tolerances) and reproductive data to determine (i) whether *N. inconspicua* is a genetically, morphologically and reproductively homogeneous species; (ii) how any physiological variation present is related to morphological and genetic diversity; (iii) the relationship of *N. inconspicua* to other *Nitzschia* species.

MATERIALS AND METHODS

Cell culture and microscopy. 36 strains of *N. inconspicua* and 21 strains of other *Nitzschia* species and one *Tryblionella* species (previously included in *Nitzschia*) were isolated

from samples from different localities of the Ebro Basin in Catalonia, Spain. Samples were collected from freshwater systems (such the lower Ebro River and the Set River, a tributary of the Ebro), brackish systems (e.g. Ebro Estuary and Ebro Delta coastal lagoons) as well as hypersaline coastal environments (e.g. salt works). *N. inconspicua* appeared to be a frequent and abundant species in the ecosystems sampled (assessed visually), especially in the Ebro Estuary, where it has been previously recorded as a common species (Rovira et al. 2009, 2012a, b). Details of each isolate are summarized in Table 1 and Supplementary Table S1. Monoclonal cultures were obtained by micropipetting single cells into Petri dishes containing MBL (Nichols 1973), WC (Guillard and Lorenzen 1972), f/2 (Guillard and Ryther 1962) or R medium (Roshchin 1994, Chepurnov and Mann 1997), depending on the salinity of the environment from which the strains were obtained (Table 1). All isolates were maintained in an incubator at 20ºC, under c. 50 µmol photons m^{-2} s^{-1} from cool-white fluorescent lights with a 24 h light period.

Frustules were cleaned following Sato et al. 2013 (in press), using a domestic drain cleaner Parozone (Leyes, Norfolk, UK) to remove organic matter. Cleaned valves were permanently mounted in Naphrax© (refractive index 1.74) and examined using a Zeiss Axio Imager M2, using a plan-apochromat 100× objective (N.A. 1.4) with bright field optics and AxioCam HRc camera. For SEM, cleaned material was pipetted onto coverslips and air-dried overnight, mounted on aluminium stubs, coated with platinum for 2 min in an Emitech K575X sputter coater (Quorum Technologies, East Grinstead, Sussex, UK), and examined using a LEO Supra 55VP Field Emission SEM (Carl Zeiss SMT) operated at 5kV (usually 4–6 mm working distance; aperture 20 mm). Images were captured as 2048 × 1536 or 3072 × 2304 pixel TIFF files.

For morphometric analyses, length, width, stria density and fibula density were measured from digital pictures for at least two isolates sharing the same genotype, when available. Three types of cells were usually measured for each isolate: (i) Small cells in cultures capable of auxosporulation, (ii) initial cells (i.e., the first cell formed within the auxospore) and (iii) enlarged cells derived from initial cells. However, due to the highly variable and irregular morphology of many of the small and initial cells (Mann et al. 2013), only measurements of large cells (category iii) were used in statistical analyses. Variation in stria densities was analysed by

ANOVA and *post hoc* Tukey–Kramer tests, using Minitab v. 16 (State College, Pennsylvania, USA).

Salinity experiments. Two isolates from each *N. inconspicua* genotype were inoculated under different salinity regimes (from 0.26 ppt to ca 35 ppt) obtained after mixing different percentages of MBL and f/2 (Table 3). F/2 medium was prepared using filtered natural sea water (ca 35 ppt) from Alfacs Bay (Mediterranean Sea). Isolates were grown in illuminated cabinets at 20°C under a cool-white fluorescent light intensity of 75 µmol photons m^{-2} s^{-1} and a 12:12 h light period. Two replicates from each isolate and treatment were assessed visually for growth with a Leitz Labovert inverted microscope at weekly intervals. After 21 days of incubation, growth was assessed, again visually, by two independent observers and these assessments are the results presented here. Growth failure happened only in freshwater conditions (0.26 ppt, MBL) and only in the case of isolates originally maintained in f/2 (ca 35 ppt) medium. In order to be sure that this was not due to a lack of acclimation, another set of salinity experiments was performed for the isolates that failed to grow in freshwater medium (0.26 ppt), this time with the strains pre-adapted for one week to lower salinity conditions (14 ppt) than those in which the isolate had originally been maintained (ca 35 ppt).

DNA extraction, amplification and sequencing. DNA was extracted from each isolate during exponential growth phase using a DNeasy Plant Kit (Qiagen, Crawley, UK). *Rbc*L and partial LSU were used for phylogenetic analyses because of their proven utility in *Nitzschia* (Trobajo et al. 2006, 2009, 2010) and other diatoms (Mann and Evans 2007; Theriot et al. 2010 for *rbc*L); they are also currently recommended for use as diatom barcodes (Mann et al. 2010; Hamsher et al. 2011). *Rbc*L (~1400 bp) was amplified using two primers, DPrbcL1 (5′-AAGGAGAAATHAATGTCT-3′) and DPrbcL7 (5′-AARCAACCTTGTGTAAGTCTC-3′) (Daugbjerg and Andersen 1997). Partial LSU (including the D1–D3 hypervariable domains, ~800 bp) was amplified with the following primers: D1R (forward: 5′-ACCCGCTGATTTAAGCATA-3′) (Scholin et al., 1994) and D3R (reverse: 5′ -TCGGAGGGAACCAGCTACTA-3′) (Nunn et al. 1996). PCR reaction volumes were 25 µL and contained 1 µL of DNA template, 2.5 µL of 10× NH4 buffer, 2.5 µL of 50 mM $MgCl_2$, 2.5 µL of 2 mM dNTPs, 0.75 µL of 10 µM forward and reverse primers, and 0.125 µL of Taq polymerase. PCR conditions for *rbc*L region were an initial denaturing phase for 3 min (94ºC), followed by 34 cycles of 94ºC for 1 min, 55ºC for 1 min and 72ºC for 1.5

min, with a final extension of 72ºC for 5 min. For LSU, PCR conditions included one initial denaturation of 94°C for 4 min, followed by 35 cycles each consisting of 1 min at 94ºC, 40 sec at 56ºC and 1 min at 77ºC and a final extension of 7 min at 72ºC. PCR products were purified with ExoSAP-IT (GE Healthcare Life Sciences, Little Chalfont, UK) and sequenced in a PCR cycler using a Big Dye terminator v. 31 sequencing chemistry (Applied Biosystems, CA, USA). Sequencing PCR reaction volumes were 10 µL and contained 1 µL of DNA template, 2 µL of 5× sequencing buffer, 0.32 µL of 10 µM sequencing primers and 0.5 µL of Big Dye terminator. Sequencing primers used in *rbc*L were the PCR primers mentioned above plus two internal primers: NDrbcL5 (5′-CTCAACCATTYATGCG-3′) and NDrbcL11 5′-CTGTGTAACCCATWAC-3′) (Daugbjerg and Andersen 1997). For LSU, along with the PCR primers (D1R and D3R), one sequencing primer D2C (reverse: 5′-CCTTGGTCCGTGTTTCAAGA-3′) (Scholin et al. 1994) was used.

*Rbc*L and LSU sequences were assembled with Sequencher 4.8 and aligned together with public sequences retrieved from Genbank (Table 1, Supplementary Table S2) using MUSCLE implemented in MEGA5 (Tamura et al. 2011). In the case of LSU, alignment was refined by eye comparing with the secondary structural model of the D1–D3 LSU rDNA region of the bryophyte *Funaria hygrometrica* (accession number: X74114) (Capesius and Van de Peer 1997) and the diatom species *Navicula cryptocephala* (accession number: FN397590) (Poulíčková et al. 2010). Finally, the ambiguously aligned positions from the most variable parts were excluded.

A total of 136 *rbc*L and 161 LSU sequences were trimmed at the ends and identical sequences were excluded, resulting in a final alignment of 83 unique *rbc*L sequences spanning 1355 bp and 92 LSU sequences spanning 838 bp. For *rbc*L, a few sequences showed a very few gaps at the ends, with the exception of *Nitzschia* sp. culture CCMP1532 (accession number: HQ337578), which only included the *rbc*L-3P region (698 bp). However, we decided to include it in phylogenetic analyses due to its morphological and genetic relationship to the *N. inconspicua* complex. Although most LSU sequences covered the D1–D3 hypervariable domains, some only comprised D1–D2 and a few (e.g. *Nitzschia* sp. culture CCMP1532, accession number: HQ396844 and *N. inconspicua* G2_1–G2_6) included only the D2–D3 region.

Genetic diversity and phylogenetic analyses. P-distances of both *rbc*L and LSU sequences were calculated by MEGA5. Each base change within indels was considered as a separate mutation.

All phylogenetic trees were rooted with *Bacillaria paxillifera* according to its basal position within the Bacillariales (Ruck and Theriot 2011). Neighbor-joining (NJ) and Maximum Parsimony (MP) were performed using MEGA5, whereas Maximum Likelihood (ML) was carried out using RAxML v. 7.2.8 (Stamatakis 2006). For NJ, 1000 bootstrap replications were performed with MEGA in that Maximum Composite likelihood distances was selected with a gamma distribution parameter obtained using jModeltest v. 0.1.1 (Posada 2008): 0.6450 for *rbc*L and 0.2030 for LSU.

For MP, bootstrap values were obtained with the Max-mini branch and bound method for 1000 replicates. In order to determine the model of sequence evolution appropriate for ML analyses, the Akaike Information Criterion (AIC) was used in jModeltest v. 0.1.1 (Posada 2008). Models and parameters were as follows. For *rbc*L: GTR+I+G, A = 0.2849, C = 0.1371, G = 0.1873, T = 0.3907; substitution rates were A–C = 0.9189, A–G = 2.9722, A–T = 1.7870, C–G = 0.6914, C–T = 5.4096, G–T = 1.0000; the proportion of invariant sites was 0.5520 and among-site rate heterogeneity was described by a gamma distribution with a shape parameter of 0.6450. For LSU: GTR+G, A = 0.2526, C = 0.1948, G = 0.3010 and T = 0.2517; substitution rates were A–C = 0.3787, A–G = 2.1310, A–T = 0.8298, C–G = 0.7086, C–T = 4.0332, G–T = 1.0000; among-site rate heterogeneity was described by a gamma distribution with a shape parameter of 0.2030. For ML, we used an option “-f a” that tells RaxML to conduct a rapid bootstrap analysis and search for the best-scoring ML tree in one single program run. We performed 100 runs.

Barcode testing. Following the recommendations of Mann et al. (2010) and Hamsher et al. (2011), two molecular markers were tested as potential barcodes for identifying *N. inconspicua*: *rbc*L-3P (790 bp after trimming the 3′ end) and the D2–D3 region of LSU (430 bp after trimming both 3′ and 5′ ends). No additional PCR was carried out. Instead, we tested the barcodes *in silico*, by extracting the relevant regions from the whole *rbc*L and D1–D3 LSU regions (obtained as described above in “Genetic diversity and phylogenetic analyses”) and identifying the primer

binding zones by reference to Hamsher et al. (2011). Results are given in Supplementary Appendix S1.

RESULTS

Genetic diversity. The 36 *N. inconspicua* isolates from the Ebro River and delta yielded 32 *rbc*L and 34 LSU sequences; the amplification failures involved different isolates for each marker (Table 1). Six unique *rbc*L sequences were found (*N. inconspicua* genotypes G1–G6), five of which were represented by three or more isolates (Table 1, Fig. 1). One genotype (G5) was shared by 19 isolates collected from two different ecosystems (salt works and a brackish aquaculture lagoon) 4.7 km apart. Another genotype (G3) comprised four isolates collected from the same ecosystem (the lower Ebro River), but from two different locations 37 km apart (c. 60 km, if measured along the river). In addition, an *rbc*L sequence downloaded from Genbank (*Nitzschia* sp. culture CCMP 1532; accession number: HQ337578) represented a further genotype, most genetically similar to genotype G5.

Each of the *rbc*L genotypes, including that of CCMP 1532, corresponded to a single LSU genotype. In addition to the seven genotypes recorded also in *rbc*L (*N. inconspicua* G1–G6 and *Nitzschia sp.* CCMP1532), two further LSU genotypes were found, represented by one isolate each (Table 1, Fig. 1). These were genotype G7 (collected in this study) and a previously sequenced isolate *N. inconspicua* culture incons-RT10 (Genbank accession AM182195: Trobajo et al. 2006), which was isolated from the Ter Vell, an old course of the River Ter close to its mouth in the Mediterranean Sea, now containing separate pools of still, brackish water (R. Trobajo, unpublished data). *N. inconspicua* incons-RT10 was almost identical (2 bp difference) to genotype G1, recorded from the Ebro and Set Rivers in S Catalonia, over 200 km from the Ter Vell. The LSU G5 sequences comprised not only the *N. inconspicua* sequences obtained in this study (Table 1), but also one sequence from Genbank labelled as '*N. frustulum*' (accession number: AF417671, from UTEX clone 2042), which was isolated from La Jolla, California (Lundholm et al. 2002).

TABLE 1. List of the 59 new isolates sequenced for this study (in bold), together with some isolates previously sequenced for other studies for which new sequences were obtained. Isolate identifier correspond to designation of the strains in Genbank database. Original isolate identifier corresponds to the isolate names in the voucher slides held in Royal Botanic Garden of Edinburgh. Other isolate designations are specified within brackets. Brief locality information (more detail is provided in Supplementary Table 1), the original salinity of the environment from which the clones were isolated (freshwater and brackish refers to habitats the exact ppt of which are unknown), the size restitution pattern (our observations) and Genbank accession numbers for *rbc*L and LSU D1/D3 are given (new accession numbers in bold). Ap = apparent apomictic auxosporulation, P = paedogamy (cases where paedogamy was documented under both LM and SEM are marked by *), and VE = vegetative enlargement. n.o. = not observed.

Taxon	Isolate identifier	Original isolate identifier	*rbc*L/ LSU *N. inconspicua* genotype	Habitat and locality	Original Salinity (ppt)	Size restitution	*rbc*L	LSU
N.* cf. *aequorea	DM1004CAT	DM1004CAT		La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	39.14	n.o.	**HF675062**	**HF679146**
N. amphibia[3]	amphibia-RT5	amphibia-RT5		Artificial stream, Royal Botanic Garden Edinburgh, UK	freshwater	n.o.	**HF675118**	AM182194
N.* cf. *ardua	L44	L44		Alfacada Bay, Ebro Delta, Catalonia, Spain	n.d.	n.o.	**HF675061**	**HF679147**
N. cf. *bulnheimiana*[a†]	AG	AG1 (207)		Pond in Estancia el Bagual, Formosa, Argentina	freshwater	n.o.	**HF675063**	AM183586
N. capitellata[a]	Capitellata Spain	Terri F222 (262)		Terri stream, Cornella de Terri, Catalonia, Spain	freshwater	n.o.	FN557032	AM909631
N. capitellata[b]	Capitellata Scot1	Nit54		Loch of Forfar, Scotland, UK	n.d.	n.o.	FN557030	**HF679148**
N. capitellata[b]	Capitellata Scot2	Nit55		Loch of Forfar, Scotland, UK	n.d.	n.o.	FN557031	**HF679149**
N. cf. *fonticola*[b]	cf fonticola1	NIT331TM		Threipmuir reservoir, near Edinburgh, UK	freshwater	n.o.	**HF675064**	**HF679150**
N. cf. *fonticola*[b]	cf fonticola2	NIT328TM		Threipmuir reservoir, near Edinburgh, UK	freshwater	n.o.	**HF675065**	**HF679150**
N. fonticola[c]	A-RT24	A-RT24		River Ter, Girona, Catalonia, Spain	freshwater	P	**HF675066**	AM182191
N. fonticola[c]	B-RT25	B-RT25		Floating Harbour, Bristol, UK	n.d.	P	**HF675067**	AM182192
N. fonticola[c]	C-RT26	C-RT26		Pond, Royal Botanic Garden Edinburgh, UK	freshwater	P	**HF675068**	AM182193
N. frustulum	Nit24	Nit24		Es Mercadal Stream, Menorca Island, Spain	0.5	n.o.	**HF675069**	–

Taxon	Isolate identifier	Original isolate identifier	*rbc*L/ LSU *N. inconspicua* genotype	Habitat and locality	Original Salinity (ppt)	Size restitution	*rbc*L	LSU
N. frustulum	Nit25	Nit25		Es Mercadal Stream, Menorca Island, Spain	0.5	n.o.	**HF675070**	–
N. inconspicua	G1_1	L52	G1	Lower Ebro River, by Ascó, Catalonia, Spain	0.52	VE	**HF675071**	–
N. inconspicua	G1_2	L54	G1	Set River, by Albagés, Catalonia, Spain	0.73	VE	**HF675072**	**HF679152**
N. inconspicua	G1_3	L55	G1	Set River, by Albagés, Catalonia, Spain	0.73	VE	**HF675073**	**HF679153**
N. inconspicua	G2_1	L46	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675074**	**HF679154**
N. inconspicua	G2_2	L47	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675075**	**HF679155**
N. inconspicua	G2_3	L48	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675076**	**HF679156**
N. inconspicua	G2_4	L49	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675077**	**HF679157**
N. inconspicua	G2_5	L50	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675078**	**HF679158**
N. inconspicua	G2_6	L51	G2	Set River, by Albagés, Catalonia, Spain	0.73	P	**HF675079**	**HF679159**
N. inconspicua	G3_1	L53	G3	Lower Ebro River, by Ascó, Catalonia, Spain	0.52	P	**HF675080**	–
N. inconspicua	G3_2	L58	G3	Lower Ebro River, by Xerta, Catalonia, Spain	0.53	P	**HF675081**	**HF679160**
N. inconspicua	G3_3	L61	G3	Lower Ebro River, by Xerta, Catalonia, Spain	0.53	P*	**HF675082**	**HF679161**
N. inconspicua	G3_4	L62	G3	Lower Ebro River, by Xerta, Catalonia, Spain	0.53	P*	**HF675083**	**HF679162**
N. inconspicua	G4_1	L4 (IRTA4)	G4	Ebro Estuary, Ebro Delta, Catalonia, Spain	31.26	P	**HF675084**	**HF679163**
N. inconspicua	G4_2	L5 (IRTA5)	G4	Ebro Estuary, Ebro Delta, Catalonia, Spain	31.26	P*	**HF675085**	**HF679164**
N. inconspicua	G4_3	L6 (IRTA6)	G4	Ebro Estuary, Ebro Delta, Catalonia, Spain	31.26	P*	**HF675086**	**HF679165**
N. inconspicua	G5_1	DM1002cat	G5	La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	39.19	P*	**HF675087**	**HF679166**
N. inconspicua	G5_2	DM1005cat	G5	La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	40.17	P*	**HF675088**	**HF679167**
N. inconspicua	G5_3	L7 (IRTA7)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P*	**HF675089**	**HF679168**

Taxon	Isolate identifier	Original isolate identifier	*rbc*L/ LSU *N. inconspicua* genotype	Habitat and locality	Original Salinity (ppt)	Size restitution	*rbc*L	LSU
N. inconspicua	G5_4	L8 (IRTA8)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675090**	**HF679169**
N. inconspicua	G5_5	L9 (IRTA9)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675091**	**HF679170**
N. inconspicua	G5_6	L10 (IRTA10)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675092**	**HF679171**
N. inconspicua	G5_7	L11 (IRTA11)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P*	–	**HF679172**
N. inconspicua	G5_8	L12 (IRTA12)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675093**	**HF679173**
N. inconspicua	G5_9	L13 (IRTA13)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675094**	**HF679174**
N. inconspicua	G5_11	L26 (IRTA26)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	–	**HF679175**
N. inconspicua	G5_12	L27 (IRTA27)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675095**	**HF679176**
N. inconspicua	G5_13	L28 (IRTA28)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675096**	**HF679177**
N. inconspicua	G5_14	L29 (IRTA29)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675097**	**HF679178**
N. inconspicua	G5_15	L30 (IRTA30)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675098**	**HF679179**
N. inconspicua	G5_16	L31 (IRTA31)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675099**	**HF679180**
N. inconspicua	G5_17	L33 (IRTA33)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	–	**HF679181**
N. inconspicua	G5_18	L34 (IRTA34)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675100**	**HF679182**
N. inconspicua	G5_19	L35 (IRTA35)	G5	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	31.26	P	**HF675101**	**HF679183**
N. inconspicua	G6_1	DM 950cat	G6	El Clot lagoon , Ebro Delta, Catalonia, Spain	4.88	P	**HF675102**	**HF679184**
N. inconspicua	G7_1	DM 948cat	G7	IRTA aquaculture lagoon, Ebro Delta, Catalonia, Spain	14.37	P*	–	**HF679185**
N.* cf. *microcephala	L56	L56		Lower Ebro River, by Flix, Catalonia, Spain	0.52	n.o.	**HF675103**	**HF679186**
N. palea	L15	L15 (IRTA15)		Lower Ebro River, by Ginestar Island, Catalonia, Spain	0.33	n.o.	**HF675104**	**HF679187**

Taxon	Isolate identifier	Original isolate identifier	*rbc*L/ LSU *N. inconspicua* genotype	Habitat and locality	Original Salinity (ppt)	Size restitution	*rbc*L	LSU
N. palea	L16	L16 (IRTA16)		Lower Ebro River, by Ginestar Island, Catalonia, Spain	0.33	n.o.	–	**HF679188**
N. palea	L17	L17 (IRTA17)		Lower Ebro River, by Ginestar Island, Catalonia, Spain	0.33	n.o.	**HF675105**	**HF679189**
N. palea	L18	L18 (IRTA18)		Lower Ebro River, by Ginestar Island, Catalonia, Spain	0.33	n.o.	–	**HF679190**
N. palea	L19	L19 (IRTA19)		Lower Ebro River, by Ginestar Island, Catalonia, Spain	0.33	n.o.	**HF675106**	**HF679191**
N. palea	laia46	laia46		Lower Ebro River, by Mora Island, Catalonia, Spain	n.d.	n.o.	**HF675107**	–
N. palea[b]	Belgium 4	Victor 02-9G (243)		Wastewater treatment plant, Destelbergen, Ghent, Belgium	freshwater	n.o.	FN557029	**HF679202**
N. palea[a]	Japan D2	Jp037-O7 (265)		Downstrean Sakura River, Tokyo, Japan	freshwater	n.o.	**HF675125**	AM183231
N. palea[a]	Japan F	Mayama (235)		Stream, Okinawa island, Japan	freshwater	n.o.	**HF675123**	AM183233
N. palea[b]	New Scot1	Nit 144D		Dunsapie Loch, Edinburgh, UK	freshwater	n.o.	**HF675126**	**HF679200**
N. palea[b]	New Scot2	Nit 335Tm-16		Threipmuir reservoir, near Edinburgh, UK	freshwater	n.o.	**HF675128**	**HF679203**
N. palea[b]	New Spain1	Ter2B1		River Ter, near Girona, Spain	freshwater	n.o.	–	**HF679192**
N. palea[b]	New Spain2	RieraSMV		Sant Martí Vell stream, near Girona, Spain	freshwater	n.o.	**HF675127**	**HF679201**
N. palea[a]	Spain A1	Nit B1 (208)		Pond, Cala Castell, Girona, Spain	brackish	n.o.	**HF675120**	AM183243
N. palea[a]	Spain A2	(Nit B2238)		Pond, Cala Castell, Girona, Spain	brackish	n.o.	**HF675124**	AM183245
N. palea[a]	SriLanka1	SLA (219)		Pond, Dambulla Rock Temple, Sri Lanka	freshwater	n.o.	**HF675121**	AM183235
N. palea[a]	SriLanka2	SLB (234)		Puddle, Dambulla, Sri Lanka	freshwater	n.o.	**HF675122**	AM183236
N. cf. *pusilla*[d]	CCMP558	CCMP558		Rock pool in Prospect, Nova Scotia, Canada	marine	Probably P	**HF675129**	**HF679204**
N.* cf. *pusilla	Nit 44	Nit 44		Threipmuir Reservoir, near Edinburgh, UK	freshwater	n.o.	**HF675119**	**HF679199**
N. pusilla	L1	L1 (IRTA1)		La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	37.32	n.o.	**HF675108**	**HF679193**

Taxon	Isolate identifier	Original isolate identifier	*rbc*L/ LSU *N. inconspicua* genotype	Habitat and locality	Original Salinity (ppt)	Size restitution	*rbc*L	LSU
N. pusilla	L2	L2 (IRTA2)		La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	37.32	n.o.	–	**HF679194**
N. pusilla	L3	L3 (IRTA3)		La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	37.32	n.o.	**HF675109**	**HF679195**
N. pusilla	L25	L4 (IRTA25)		La Trinitat salt works pond, Ebro Delta, Catalonia, Spain	37.32	n.o.	**HF675110**	**HF679196**
N. soratensis	DM1008MK	DM1008		Houselop Beck, Co. Durham, UK	0.26	Ap	**HF675111**	**HF679197**
N. soratensis	DM1009MK	DM1009		Houselop Beck, Co. Durham, UK	0.26	Ap	**HF675112**	**HF679198**
N. soratensis	NitMK_A1	MK_A1		Houselop Beck, Co. Durham, UK	0.26	Ap	**HF675113**	–
N. soratensis	NitMK_A2	MK_A2		Houselop Beck, Co. Durham, UK	0.26	Ap	**HF675114**	–
N. soratensis	NitMK_C	MK_C		Houselop Beck, Co. Durham, UK	0.26	Ap	**HF675115**	–
***Nitzschia* sp.**	s0819	s0819		Trabucador Beach, Ebro Delta, Catalonia, Spain	32.9	n.o.	**HF675116**	–
***Tryblionella* sp.**	s0863	s0863		Buda island, Ebro Delta, Catalonia, Spain	29.9.	n.o.	**HF675117**	–

[a] Clones isolated for Trobajo et al. (2009). [†] *N.* cf. *bulnheimiana* was originally (Trobajo et al. 2009) identified as *N.* cf. *frustulum*. A re-examination of the voucher slide has concluded that its closest morphological resemblance is *N. bulnheimiana*, and therefore, we refer it here as *N.* cf. *bulnheimiana*.

[b] Clones isolated for Trobajo et al. (2010)

[c] Clones isolated for Trobajo et al. (2006)

[d] *N.* cf. *pusilla* CCMP558 was originally referred to as *N. frustulum* (Hamsher et al. 2011). However, our SEM re-examination revealed that culture CCMP558 cannot be assigned to *N. frustulum* since its stria density (ca 40 in 10µm) is much higher than in *N. frustulum* (ca 27 in 10µm, Trobajo et al. 2013), and its raphe is not centrally interrupted by an small central nodule, as it is the case of *N. frustulum* (Trobajo et al. 2013). It closest morphological resemblance is *N. pusilla* Grunow and therefore, we refer it here as *N.* cf. *pusilla*.

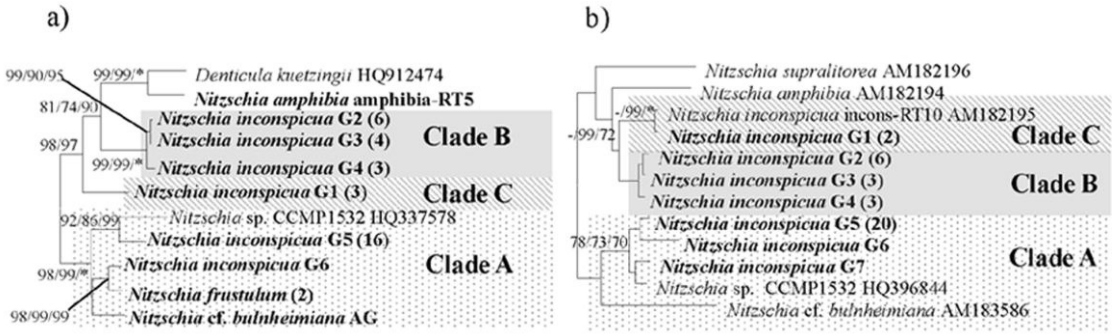


FIG. 1. Molecular phylogeny of *N. inconspicua* isolates inferred from *rbc*L (a) and LSU (b). Only bootstrap values >70% for NJ/MP/ML are shown and bootstraps of 100% are indicated by an "*". Numbers in brackets give the number of isolates that shared the same genotype. Genotypes sequenced for this study are highlighted in bold. The figure is an enlarged section of the molecular phylogeny of Bacillariaceae (Fig. 2).

Analysis of P-distances. The seven *N. inconspicua rbc*L genotypes differed by 0.1–7.2% (1–81 bp) (Supplementary Table S3), with the lowest P-distance (0.1%, only 1 bp) between genotypes G2 and G3 of *N. inconspicua*, both belonging to clade B. The highest distances (7.2%, 81 bp) were between genotypes G4 (clade B) and G5 (clade A). Distances between genotypes belonging to different *N. inconspicua* clades were of the same order as, or in some cases greater than, distances between *N. inconspicua* and phylogenetically closely related diatoms that differed morphologically and were identifiable as separate species (*N. frustulum*, *N.* cf. *bulnheimiana*, *N. amphibia*, *N.* cf. *aequorea* and *D. kuetzingii*). LSU P-distances among the nine *N. inconspicua* genotypes were comparable to *rbc*L distances, with a range of 0.4–9.7% (Supplementary Table S4). P-distances among *N. inconspicua* clades were a little lower than, but comparable to, distances between any *N. inconspicua* clade and phylogenetically close species (*N. amphibia, N.*cf. *bulnheimiana,* and *N. supralitorea*).

*Relationships among N. inconspicua genotypes. Rbc*L analyses revealed that the *N. inconspicua* genotypes belonged to three clades, each with high bootstrap support (≥ 99% in ML) (Fig. 1). Clade A grouped *N. inconspicua* genotypes G5 and G6 and *Nitzschia* sp. CCMP1532, together with *N. frustulum* and *N.* cf. *bulnheimiana*. Clade B contained *N. inconspicua* genotypes G2–G4, which formed a very well supported subclade (ML 100%) that was sister to a well supported subclade (ML 90%) containing *N. amphibia* and *D. kuetzingii.* Finally, Clade C comprised a single *N. inconspicua* genotype, G1.

Clades A–C were also recovered in the LSU phylogenetic tree, but with no or low bootstrap support (< 70% in most cases) (Fig. 1). There was moderate support for the *N. inconspicua* subclade within Clade A, which also contained *Nitzschia* sp. CCMP1532 (as in the *rbc*L tree), and the G7 genotype of *N. inconspicua* (for which no *rbc*L sequence was obtained). Not surprisingly, given the very high LSU sequence identity between them, *N. inconspicua* genotype G1 and the previously sequenced incons-RT10 clone of *N. inconspicua* formed a very well supported clade in LSU.

Phylogeny of Bacillariaceae. Relationships between *N. inconspicua* and other Bacillariaceae are illustrated in Fig. 2. In both *rbc*L and LSU gene trees, *N. inconspicua* isolates formed a paraphyletic grouping within a clade (well supported in *rbc*L, poorly supported in LSU) also containing *Nitzschia amphibia* and *N.* cf. *bulnheimiana.* In the *rbc*L tree, this clade also contained *Denticula kuetzingii* and *N. frustulum* and there was moderate support for a relationship with *N.* cf. *aequorea*.

At a deeper level, the two gene trees (Fig. 2) agreed in separating *Nitzschia* sect. *Lanceolatae* species into two principal groups: Group I containing *N. inconspicua* and the related species already mentioned, as well as *N. fonticola*, *N. soratensis*, *N. pusilla*, *N.* cf. *microcephala*; and Group II containing *N. palea*, *N. capitellata*, *N.* cf. *ardua* and *N.* cf. *pusilla*. However, the Group I *Lanceolatae* species were contained within a clade also containing species generally classified in other genera of Bacillariaceae, namely *Pseudo-nitzschia*, *Neodenticula*, *Fragilariopsis* and *Cylindrotheca*. The Group II *Lanceolatae* was related to *N. filiformis* (*Nitzschia* sect. Obtusae) and perhaps (no statistical support) to the genus *Psammodictyon*.

Morphology. LM photos of selected large and (where available) small valves of each of the *N. inconspicua* genotypes are shown in Fig. 3. Within isolates, cell size and shape showed high variability as a consequence of the life-cycle (Figs. 3 and 4). On the whole, whatever the genotype, large specimens were linear-lanceolate with slightly protracted and narrow apices. The smallest cells (4–6 µm long), on the other hand, were broadly lanceolate, with more rounded apices than in large cells (Figs 3 and 4). Sometimes small cells became elliptical (e.g. Figs. 3b and f; 4j and k). The only exceptions were in *N. inconspicua* G1, where some of the larger cells also had rounded apices (Figs. 3a and 4n).

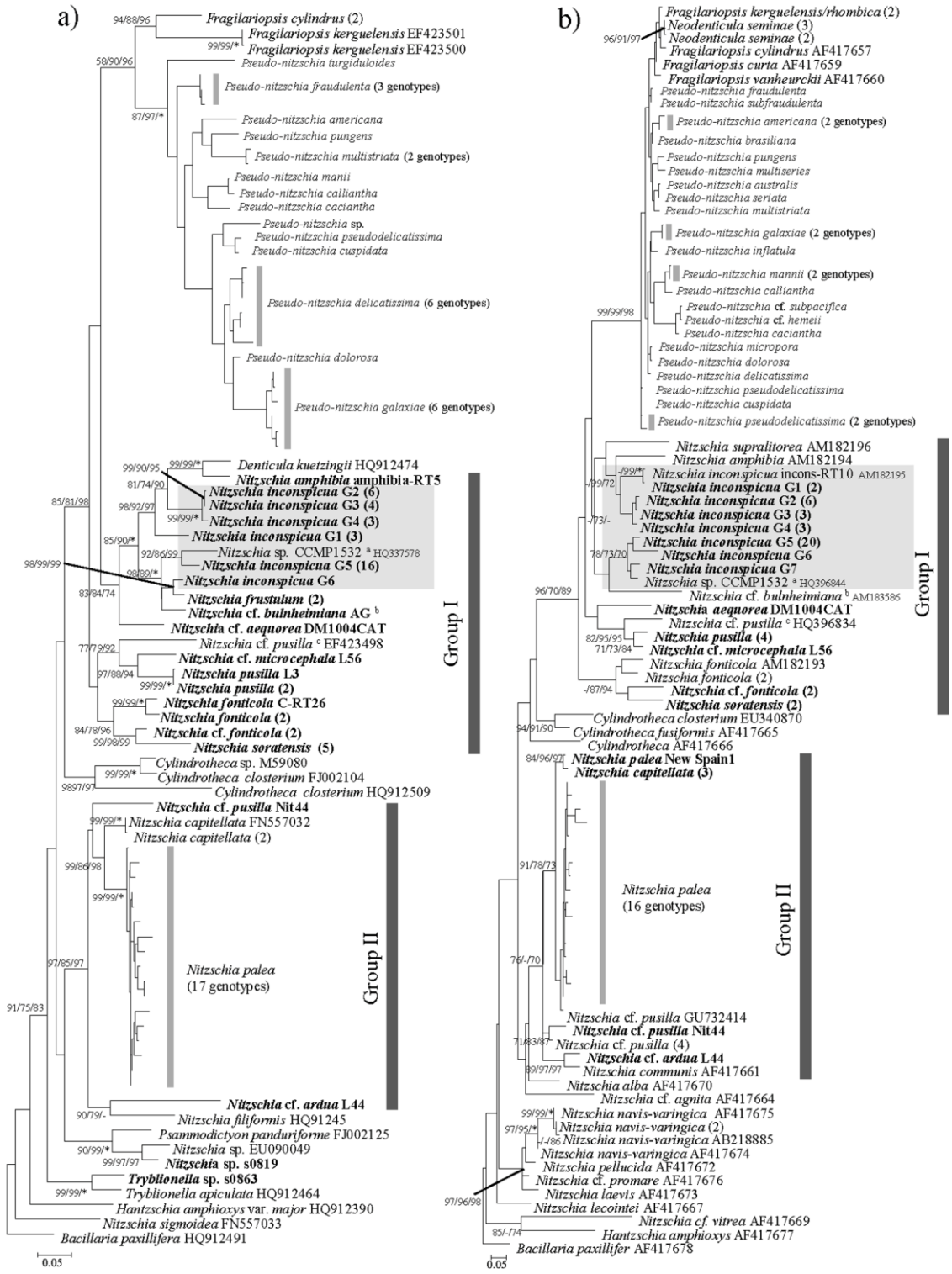


FIG. 2. Molecular phylogeny of Bacillariaceae inferred from *rbc*L (a) and LSU (b). Only bootstrap values >70% for NJ/MP/ML are shown and bootstraps of 100% are illustrated with an "*". *N. inconspicua* genotypes are highlighted in grey. Numbers in brackets mean number of isolates that shared the same genotype. Genotypes for which no accession number is given belong to isolates sequenced for this study and are highlighted in bold. Genotype labels for *N. palea* are omitted in order to simplify the tree. In addition, bootstrap values, accession numbers and number of isolates within genotypes are also omitted for *N. palea* and *Pseudo-nitzschia*. [a–c] Previously named in Genbank as: *Nitzschia* sp. (a), *N.* cf. *frustulum* (b) and *N. frustulum* (c). Groups I and II within sect. *Lanceolatae* are highlighted in dark grey bars.

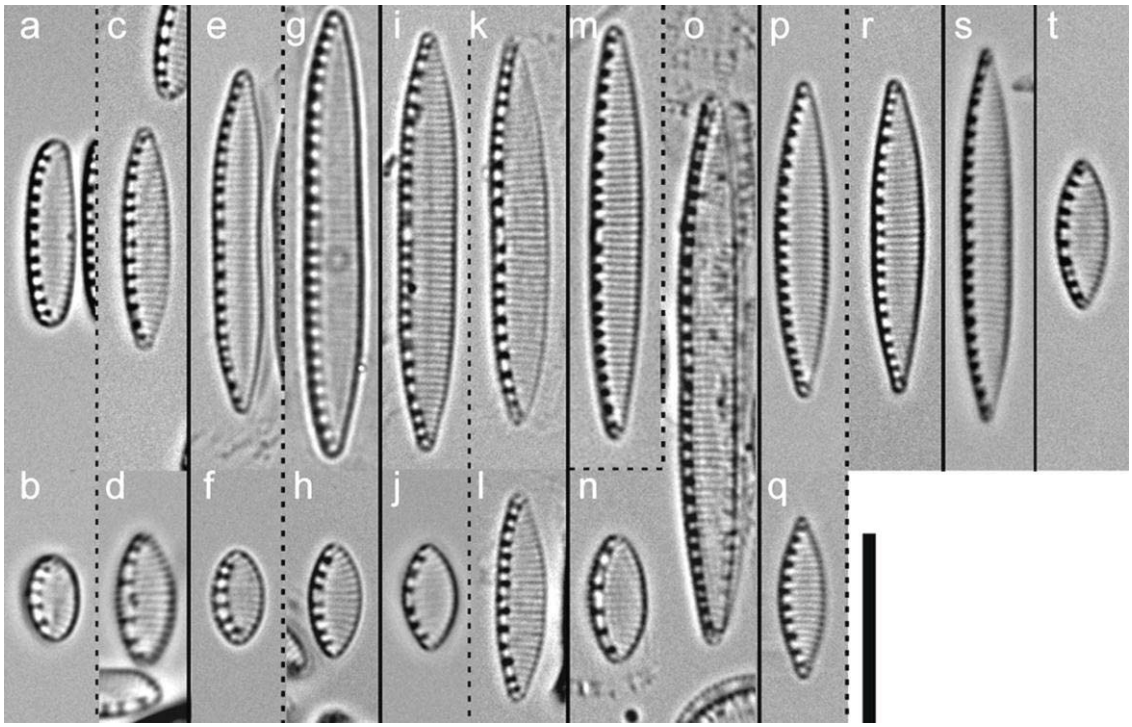


FIG. 3. Morphological variability of *N. inconspicua* in LM. Micrographs of isolates that share identical genotype for *rbc*L and/or LSU D1/D3 are grouped by solid lines. Micrographs from different isolates are separated by dashed lines. Small (parental) and large valves (post-auxosporulation or following vegetative cell enlargement) are illustrated when possible (exceptions: m, r–t). (a, b) G1_1, (c, d) G1_2, (e, f) G2_3, (g, h) G2_5, (i, j) G3_1, (k, l) G3_2, (m) G4_1 (large cell), (n, o) G4_3, (p, q) G5_13, (r) G5_1 (large cell), (s) G7_1, and (t) G6_1. Scale bar: 10μm.

Although there was significant variation among isolates in valve length, width, stria density and fibula density (ANOVA, not shown), the only parameter in which there was an obvious correlation with genotype or clade, and in which intra-isolate variation was low, was stria density (Fig. 5c). However, although the mean stria density was consistently and significantly higher in genotypes G5 and G6 (clade A) than in any of the clade B isolates measured (genotypes G2–4), the ranges overlapped (Table 2) and *post hoc* tests showed that the genotypes formed a continuum with respect to this character (Supplementary material, Appendix S2).

There seemed to be variation among clones in the maximum size of cells produced by auxosporulation (i.e. the sizes of initial cells). Thus, for example, clone G4_3 produced initial cells that were up to 30 μm or more in length, whereas in G2_3, G3_1 and G3_2 no initial cells greater than 24 μm were found (Table 2). However, the numbers of initial cells measured were small and dependence of initial cell size on clone, genotype or (within a clone) gametangial size cannot yet be analysed in detail.

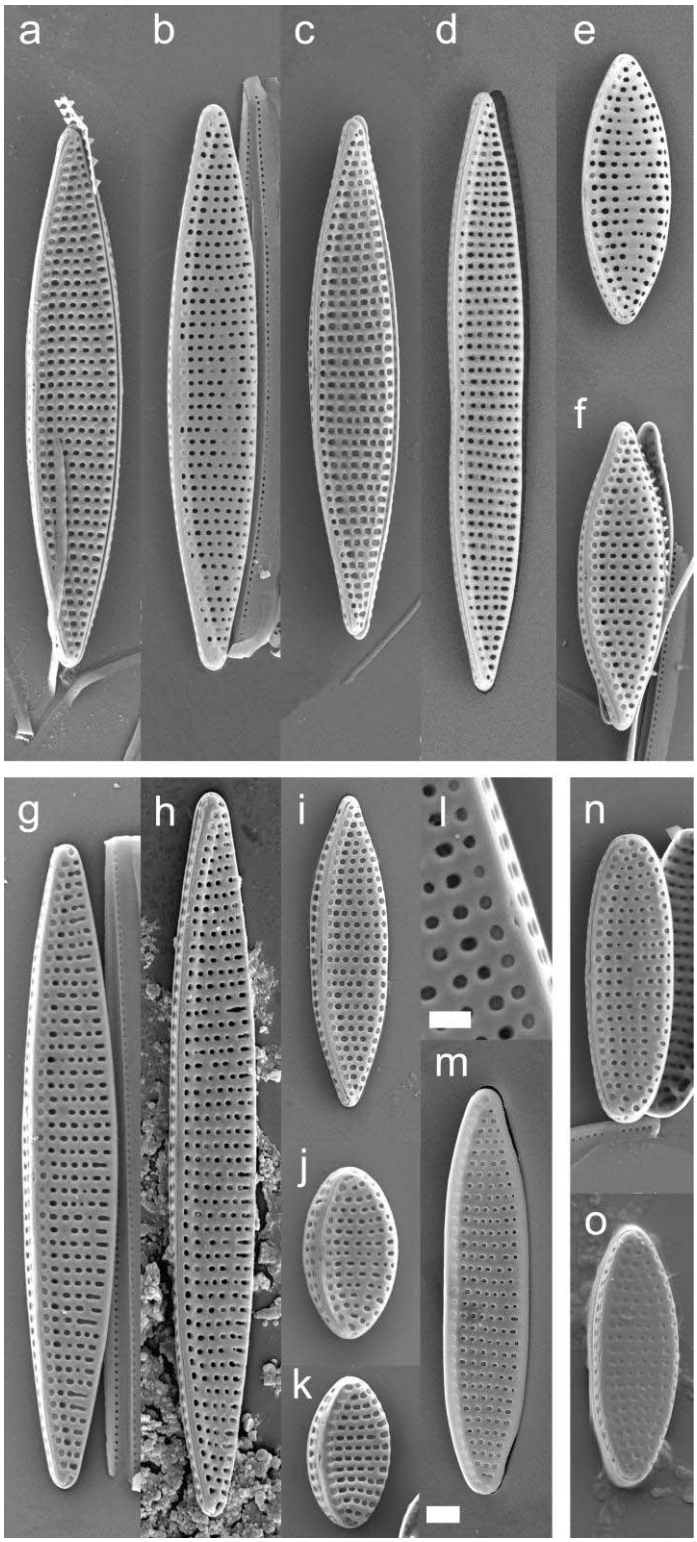


FIG. 4. SEM micrographs of *N. inconspicua* isolates showing different *rbc*L and LSU genotypes. Isolates are grouped according to the clades evident in the phylogenetic trees. (a–f) Clade A, (g–m) Clade B, and (n, o) Clade C. a: G5_8 (large cell), b: G5_2 (large cell), c: G5_13 (large cell), d: G7_1, e: G6_1, f: G5_18 (small cell), g: G4_1 (large cell), h: G4_3 (large cell), i: G4_2 (small cell), j: G2_3 (small cell), k: G2_5 (small cell), l: G4_1 (detail of areolae and central raphe endings), m: G3_4 (medium cell), n:G1_1 (medium cell), o: incons-RT10. Scale bars: 1µm except (l) (0.5 µm).

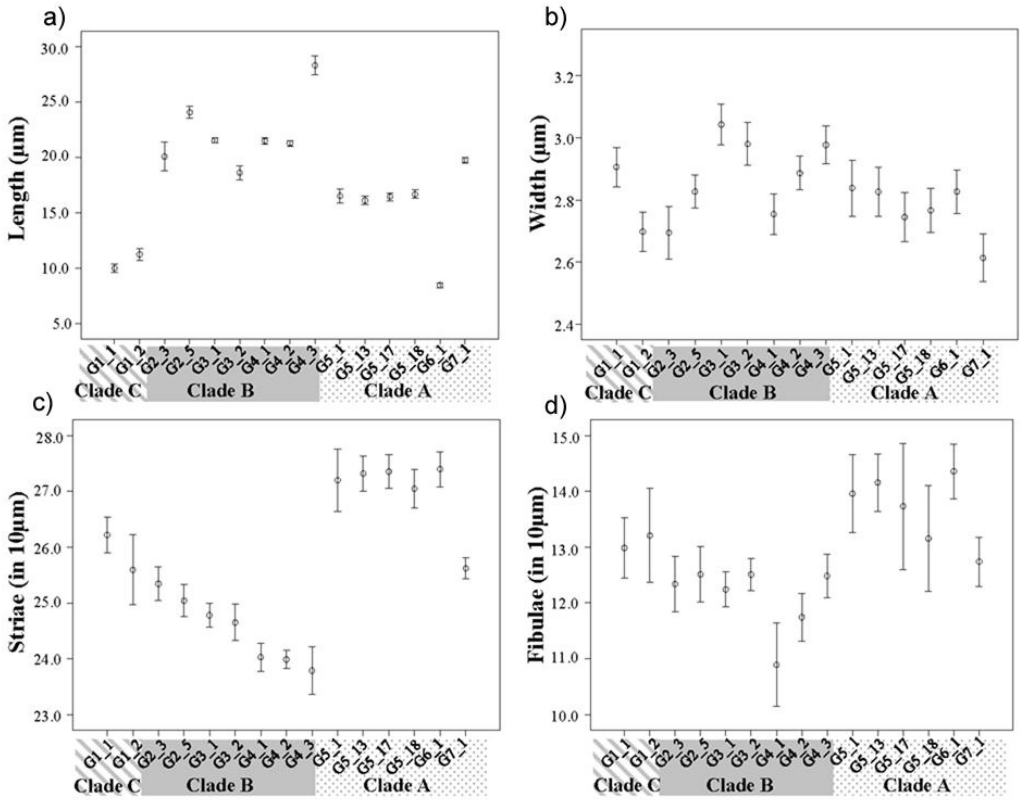


FIG. 5. Error bar graphs showing the morphological variability within *N. inconspicua*. Isolates are arranged according to genotypes (G1–G7) and highlighted according to the phylogenetic clades found: Clade A (stippled), B (grey) and C (hatched). Only larger valves found in each isolate are included (Table 2).

All *N. inconspicua* isolates had a marginal raphe system with small fibulae appearing in LM as rectangular dots (Fig. 3). The stria appeared plain in LM, or, when the optics were optimized, faintly punctate. All isolates had uniseriate striae containing simple round or elliptical pores, both on the valve face and within the raphe canal (Fig. 4); a central interruption to the raphe (with simple slightly expanded raphe endings: Fig. 4l); and hooked terminal raphe fissures (not shown: see Trobajo et al. 2013). No ultrastructural characteristics were found to separate genotypes or clades. We did not obtain material of culture CCMP 1532 for morphological examination. However SEM micrographs of culture CCMP 1532 in the BOLD database (available at http://www.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=373683 under the label "*Nitzschia* sp. 2") show an ultrastructure identical to the Ebro isolates and to *N. inconspicua* type material (Trobajo et al. 2013)

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TABLE 2. Morphometric measurements for 15 *N. inconspicua* isolates and some phylogenetically related and/or morphologically similar *Nitzschia* species. Wherever possible, both large and small valves were measured for each isolate, 'large' valves being those formed during the first few cell divisions following cell enlargement (usually auxosporulation). Some of the large valves were initial valves (identified by their arched valve faces and more irregular outline), in such cases the upper limit for the length is italicized, to indicate that it is an especially reliable estimate of the upper limit for length in that lineage. 'Small' valves were the smallest formed in our cultures before cell enlargement occurred. X = average, SD = standard deviation.

Isolate	*n*	Clade	Length (*µ*m)		Width (*µ*m)		Striae (in 10 *µ*m)		Fibulae (in 10 *µ*m)	
			range	(X ± SD)	range	(X ± SD)	range	(X ± SD)	range	(X ± SD)
Large cells										
G1_1	27	C	8.5–12.2	**(10.0 ± 1.0)**	2.6–3.2	**(2.9 ± 0.2)**	24.7–28.8	**(26.2 ± 0.8)**	10.6–15.7	**(13.0 ± 1.4)**
G1_2	8	C	10.1–12.0	**(11.2 ± 0.8)**	2.6–2.9	**(2.7 ± 0.1)**	24.5–27.3	**(25.6 ± 0.9)**	11.4–15.0	**(13.2 ± 1.2)**
G2_3	15 (5)[1]	B	16.6–*23.4*	**(20.1 ± 2.5)**	2.4–3.0	**(2.7 ± 0.2)**	24.0–26.3	**(25.3 ± 0.6)**	9.7–13.4	**(12.3 ± 1.0)**
G2_5	16 (8)[1]	B	22.9–*26.1*	**(24.1 ± 1.1)**	2.7–3.1	**(2.8 ± 0.1)**	23.9–25.9	**(25.0 ± 0.6)**	10.5–13.7	**(12.5 ± 1.0)**
G3_1	37 (8)[1]	B	20.0–*22.8*	**(21.5 ± 0.7)**	2.7–3.5	**(3.0 ± 0.2)**	23.0–25.9	**(24.8 ± 0.7)**	10.5–14.3	**(12.2 ± 1.0)**
G3_2	37 (2)[1]	B	15.8–*22.3*	**(18.6 ± 1.9)**	2.6–3.5	**(3.0 ± 0.2)**	22.8–28.0	**(24.6 ± 1.0)**	10.7–14.5	**(12.5 ± 0.9)**
G4_1	15	B	20.3–22.3	**(21.5 ± 0.6)**	2.7–3.0	**(2.9 ± 0.1)**	22.9–24.8	**(24.0 ± 0.5)**	7.7–12.7	**(10.9 ± 1.4)**
G4_2	20	B	20.1–22.4	**(21.3 ± 0.5)**	2.7–3.2	**(2.9 ± 0.1)**	23.8–24.8	**(24.0 ± 0.4)**	9.9–13.2	**(11.7 ±1.0)**
G4_3	8 (8)[1]	B	26.6–*30.3*	**(28.3 ± 1.2)**	2.9–3.1	**(3.0 ± 0.1)**	23.0–24.7	**(23.8 ± 0.6)**	11.9–13.4	**(12.5 ± 0.6)**
G5_1	10	A	14.1–17.8	**(16.5 ± 1.0)**	2.6–3.2	**(2.8 ± 0.1)**	26.5–29.4	**(27.2 ± 0.9)**	12.4–15.5	**(14.0 ± 1.1)**
G5_13	12 (1)[1, 2]	A	15.3–*17.4*	**(16.1 ± 0.6)**	2.6–3.1	**(2.8 ± 0.1)**	26.5–28.3	**(27.3 ± 0.5)**	12.8–15.5	**(14.5 ± 0.9)**
G5_17	9	A	15.8–17.4	**(16.4 ± 0.5)**	2.5–2.9	**(2.7 ± 0.1)**	26.5–27.9	**(27.4 ± 0.4)**	11.5–16.5	**(13.7 ± 1.7)**
G5_18	6	A	16.1–17.4	**(16.7 ± 0.5)**	2.7–2.9	**(2.8 ± 0.1)**	26.4–27.4	**(27.0 ± 0.4)**	11.2–14.5	**(13.2 ± 1.2)**
G6_1	11	A	7.8–8.8	**(8.4 ± 0.3)**	2.6–3.0	**(2.8 ± 0.1)**	26.6–28.6	**(27.4 ± 0.5)**	13.0–15.5	**(14.4 ± 0.8)**
G7_1	19	A	18.9–20.8	**(19.7 ± 0.5)**	2.5–2.9	**(2.6 ± 0.2)**	24.9–26.5	**(25.6 ± 0.4)**	10.9–14.5	**(12.7 ± 1.0)**
Small cells										
G1_1	3	C	4.6–5.1	**(4.8 ± 0.3)**	2.7–2.8	**(2.7 ± 0.0)**	25.0–28.6	**(26.9 ± 1.8)**	12.3–15.0	**(13.7 ± 1.3)**
G1_2	4	C	3.0–8.4	**(7.4 ± 0.8)**	2.8–3.3	**(3.1 ± 0.2)**	24.9–27.9	**(26.8 ± 1.3)**	11.2–15.3	**(14.1 ± 1.9)**
G2_3	25	B	4.7–6.9	**5.5 ± 0.5)**	2.6–3.1	**(2.8 ± 0.1)**	26.0–30.4	**(28.3 ± 1.0)**	11.5–17.2	**(13.5 ± 1.6)**

Small cells										
G3_1	20	B	4.8–6.5	**(5.6 ± 0.5)**	2.5–3.0	**(2.8 ± 0.1)**	26.8–30.8	**(28.4 ± 1.3)**	10.9–16.3	**(13.8 ± 1.6)**
G3_2	9	B	5.1–6.6	**(6.1 ± 0.5)**	2.7–2.9	**(2.8 ± 0.1)**	24.8–29.6	**(27.0 ± 1.7)**	11.8–15.5	**(14.0 ± 1.1)**
G4_3	17	B	5.4–9.8	**(7.9 ± 1.1)**	2.9–3.4	**(3.1 ± 0.1)**	24.7–29.1	**(26.8 ± 1.1)**	9.7–17.5	**(13.5 ± 2.0)**
G5_13	7	A	8.2–8.7	**(8.4 ± 0.2)**	2.4–2.8	**(2.6 ± 0.1)**	27.2–29.7	**(28.5 ± 0.8)**	13.6–18.0	**(15.2 ± 1.7)**
G5_17	6	A	7.5–8.2	**(7.8 ± 0.2)**	2.3–2.8	**(2.5 ± 0.2)**	28.0–29.9	**(28.6 ± 0.7)**	12.4–17.8	**(15.6 ± 1.9)**
G5_18	19	A	7.5–10.4	**(9.0 ±1.0)**	2.3–2.9	**(2.6 ± 0.2)**	27.7–30.1	**(28.5 ± 0.6)**	12.0–19.1	**(14.8 ± 2.0)**
Other Nitzschia species										
Isolate	***n***	**Clade**	**Length (*µ*m)**		**Width (*µ*m)**		**Striae (in 10 *µ*m)**		**Fibulae (in 10 *µ*m)**	
			range	**(X ± SD)**	**range**	**(X ± SD)**	**range**	**(X ± SD)**	**range**	**(X ± SD)**
N. frustulum	15	A	18.4–20.6	**(19.5 ± 0.6)**	3.2–3.7	**(3.4 ± 0.2)**	24.8–26.9	**(25.5 ± 0.6)**	10.0–15.4	**(12.0 ± 1.3)**
N. cf. *bulnheimiana*	24	A	12.6–14.6	**(13.7 ± 0.5)**	3.0–3.4	**(3.2 ± 0.1)**	22.2–23.8	**(23.2 ± 0.4)**	11.4–14.5	**(12.5 ± 1.0)**
N. cf. *aequorea*	5	–	11.7–12.8	**(12.3 ± 0.4)**	3.6–3.9	**(3.7 ± 0.1)**	[c. 36][3]	**–**	14.4–18.2	**(16.3 ± 1.4)**
N. soratensis	5	–	6.6–7.7	**(7.1 ± 0.4)**	2.8–3.1	**(2.9 ± 0.1)**	29.3–30.6	**(30.1 ± 0.5)**	9.2–12.6	**(10.2 ± 1.4)**

[1] The number in parenthesis is the number of probable initial cells among the *n* total number of large valves.

[2] Although only one initial cell was measured among the large cells of G5_13, measurements agreed with initial cells observed under SEM (data not included).

[3] Striae were not detected in LM. The figure given was obtained from SEM photographs.

Other newly isolated *Nitzschia* strains belonging to clade A in one or both of the gene trees (Fig. 1), namely *N. frustulum* (Supplementary Fig. S1, a and e) and *N.* cf. *bulnheimiana* (Supplementary Fig. S1, b and f), could be distinguished morphologically from all of the *N. inconspicua* strains (Table 2). *N. frustulum*, though exhibiting very similar valve shape and ultrastructure to *N. inconspicua* (Supplementary Fig. S1, a and e), had wider valves, in accordance with *N. frustulum* type material (Trobajo and Cox 2006, Trobajo et al. 2013). *N.* cf. *bulnheimiana* differed from all *N. inconspicua* by the ultrastructure of the striae in the raphe canal, which almost always comprised double areolae in cf. *bulnheimiana* and always single areolae in *inconspicua* (compare Supplementary Fig. S1f with Fig. 4), and also by valve width (greater in *N.* cf. *bulnheimiana*) and stria density (lower in *N.* cf. *bulnheimiana*).

Three further *Nitzschia* species belonging to the same clade as *N. inconspicua* in the *rbc*L and LSU gene trees were *N.* cf. *aequorea*, *N. amphibia* and *N. supralitorea*. *N.* cf. *aequorea* differed from *N. inconspicua* by its wider valves, lanceolate shape, slightly protracted and very narrow poles and higher stria density (ca 36 in 10 µm), and by its lack of central raphe endings, also lacking in *N. supralitorea* (Supplementary Fig. S1, g and i). *N. amphibia* could be differentiated from *N. inconspicua* by the presence of cribra in the areolae (Supplementary Fig. S1j) and the presence of double areolae (or triple areolae in some cases) in the raphe channel, as in *N.* cf. *bulnheimiana*, as well as a lower stria density.

Salinity experiments. N. inconspicua isolates exhibited slightly different salinity preferences and tolerances (Table 3), ranging from brackish/marine to truly euryhaline. Salinity preferences of the isolates were genotype specific but did not correspond with the phylogenetic clades or the morphological variation observed. For example, clade B (genetically less variable than the rest) comprised both brackish/marine and euryhaline isolates. Similarly, the isolates showing the lowest stria density (*N. inconspicua* G4_1, G4_2 and G4_3) and the highest one (*N. inconspicua* G5_1, G5_13, G5_17, G5_18) shared the same response to salinity (brackish/marine) (Fig. 5c; Table 3).

Auxosporulation pattern. Thirty-three of the 36 *N. inconspicua* isolates belonging to genotypes G2–G6 showed the same type of automictic auxosporulation, namely paedogamy (Table 1), in which a single unpaired cell produces two gametes, which fuse with each other to restore diploidy.

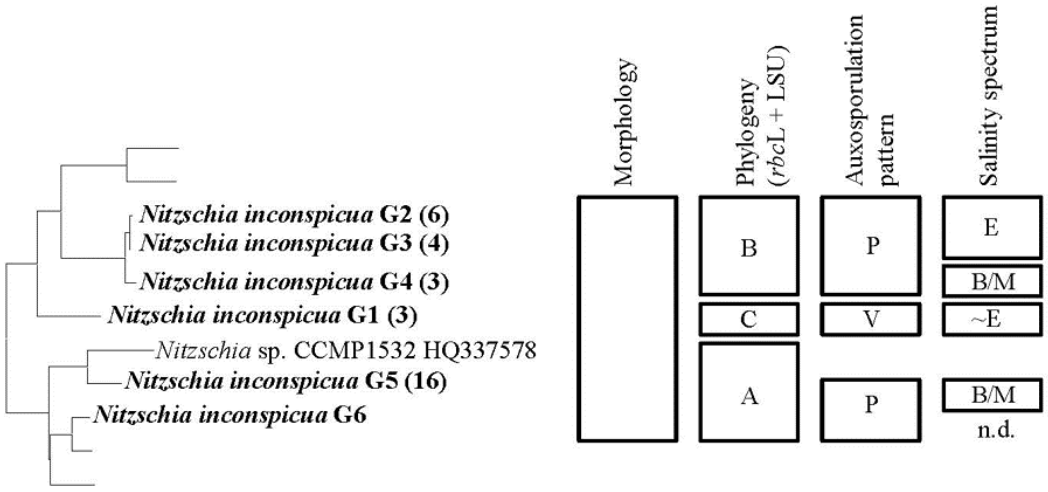

FIG. 6. Representation of *N. inconspicua* variation according to different sets of criteria. A, B, C: genotypic clades; P: paedogamic, auxosporulation, V: vegetative enlargement; B/M: brackish/marine, E: euryhaline, ~E: almost euryhaline, n.d: data not determined. Numbers in brackets indicate the number of isolates within a genotype.

The details of paedogamous auxosporulation are described for G3_3 and G3_4 by Mann et al. (2013), there referred to by their original identifiers, L61 and L62, and illustrated here for G2_4, G4_2 and clone L65 (not sequenced) in Supplementary Fig. S2. In order to test whether paedogamy was obligate, isolates were mixed in pairwise combinations but no case of allogamous pairing, either intraclonal or interclonal, was observed, so that all the 33 isolates of *N. inconspicua* G2–G6 are likely to be strictly paedogamous. Though the isolates were treated always under the same conditions (e.g. light, photoperiod, temperature), the three isolates of G1 never showed paedogamy, nor any other type of sexual reproduction during the study period (~ 2 years). Instead, they restored their cell length through vegetative cell enlargement without formation of gametes or a perizonium (not shown).

DISCUSSION

Morphological, reproductive and sequence diversity in N. inconspicua. The morphology and ultrastructure of all the *N. inconspicua* isolates agree with the description of the species provided by Trobajo et al. (2013), who examined the type material. Among the isolates there was significant variation in metric characters, but there was no clear separation of *N. inconspicua* clades and genotypes. Thus, although there were significant differences in mean stria density between some of the isolates, the differences between strains and genotypes were small and the ranges often overlapped. Overall, variation in this character is continuous.

TABLE 3. Growth of 10 *N. inconspicua* isolates after 21 days of incubation under different salinity regimes, assessed visually. Two isolates of all available genotypes were chosen, and two replicates for each isolate were inoculated and assessed. **++++** = very good, **+++** = good, **++** = moderately, **+** = sparse, @ = lack of growth but survival of the inoculum and cells grouped in clumps.

Isolate	Genotype	Habitat of isolates	f/2 (~35ppt)	30% MBL (24.5ppt)	60% MBL (14ppt)	MBL (0.26 ppt)	Salinity response
G1_1	**G1**	Ebro River	++++	++++	+++	++	**almost euryhaline**
G1_2		Set River	++++	++++	+++	++	
G2_3	**G2**	Set River	++++	++++	+++	+++	**euryhaline**
G2_5		Set River	++++	++++	+++	+++	
G3_1	**G3**	Ebro River	++++	++++	++++	+++	**euryhaline**
G3_2		Ebro River	++++	++++	++++	+++	
G4_1*	**G4**	Ebro Estuary	++++	++++	+++	+	**Brackish/marine**
G4_2*		Ebro Estuary	++++	++++	+++	@	
G5_1	**G5**	La Trinitat salt works pond	++++	++++	+++	@	**Brackish/marine**
G5_17		Brackish aquaculture lagoon	++++	++++	++++	+	

* These isolates were also studied by Trobajo et al. (2011, as "*N. frustulum*"), who found good growth at the further salinity of 9.5 ppt.

Hence, although an individual large valve of '*N. inconspicua*' with > 27 striae in 10 µm might be assigned with high confidence to clade A and individual valves with < 24.5 striae in 10 µm to clade B (particularly genotype 4), some such stria-based identifications will be wrong, while valves with intermediate striation densities could belong to any clade. In this respect *N. inconspicua* resembles the *N. palea* complex (Trobajo et al. 2009), in which there is also clinal variation in stria density, between means of 33 and 47 in 10µm. [It could be argued that some of the variation in stria density is phenotypic, reflecting growth of the isolates in different salinities, since genotype 5, with a consistently high stria density (Table 2, Fig. 5), was also the genotype grown in the highest salinity (Table 1). However, experiments indicate that lower salinity leads to higher stria density in *N. inconspicua*, not vice versa (Trobajo et al. 2011, as '*N. frustulum*').]

Most *N. inconspicua* isolates produced auxospores and did so via paedogamy, described by Mann et al. (2013). However, isolates sharing the G1 genotype (clade C) restored cell length through vegetative cell enlargement. Vegetative enlargement is known in various unrelated centric and pennate diatoms and takes place in the latter without formation of a perizonium (e.g. von Stosch 1965, Gallagher 1982, Roshchin and Chepurnov 1992, Chepurnov et al. 2004, Chepurnov and Mann 1997, Sato et al. 2008b). Perizonia are thought to play an important role in morphogenesis, constraining the expansion of the auxospore and creating the shape inherited by the initial cell and its descendants (Mann 1994). Hence absence of a perizonium in the vegetative enlargement of the G1 isolates could explain their greater morphological plasticity, with both broadly rounded and narrower protracted apices in expanded cells.

We cannot yet exclude the possibility that the G1 isolates are heterothallic and that vegetative cell enlargement is facultative since, with only three clones available for study, it is possible that the lack of a sexual response in mixed cultures was because all three clones happened to share the same mating type. However, independently of the cause of reproductive failure, the G1 isolates seem to have some specific requirements for reproduction that differ from the rest of isolates, signalling further genotypic variation within *N. inconspicua* complex beyond the LSU, *rbc*L, ecophysiological and slight morphometric differences observed.

Comparing the tree branch lengths, genetic distances between *N. inconspicua* genotypes are comparable to or greater than those between many well-accepted species of *Pseudo-nitzschia*, for both *rbc*L and LSU (Fig. 2). They are also greater than between closely related

species of *Sellaphora* (Evans et al. 2007), including reproductively isolated species within the *S. auldreekie* group (P. Vanormelingen, personal communication), and considerably greater than between putative species of *N. palea*, some of which have been shown to be reproductively isolated (Trobajo et al. 2009).

However, we should note the possibility that evolutionary rates may not be homogeneous among genes (Wolfe et al. 1987, Sorhannus and Fox 1999, Trobajo et al. 2010) or taxa (Bousquet et al. 1992, Gaut et al. 1996, Daugbjerg and Andersen 1997). Thus the higher *rbc*L and LSU genetic distances between *N. inconspicua* genotypes, relative to *Pseudo-nitzschia*, *N. palea* or *Sellaphora* species, may not reflect the same degree of divergence in other parts of the genome.

Furthermore and more importantly, the deep branchings of inbreed lineages may give a falsely high impression of their age in the phylogenetic tree. Variation in evolutionary rates for any given gene may be caused by differences in reproductive biology: outbreeding allogamous species should on average evolve less rapidly with respect to neutral characters (such as most third codon position changes, which are the majority of substitutions in *rbc*L) than inbreeding species, because of a larger effective population size (Charlesworth and Charlesworth 2010). Paedogamous automixis, which is a form of intratetrad mating (i.e. fusion of gametes or nuclei formed in a single meiosis) and thus represents extreme inbreeding (Mann et al. 2013), seems to be characteristic of *N. inconspicua* lineages (apart from genotype 1), and so their genomes might be expected to evolve relatively quickly. All *Pseudo-nitzschia* species (e.g. Amato et al. 2007, Quijano-Scheggia et al. 2009, Holtermann et al. 2010, Sarno et al. 2010), on the other hand, appear to be allogamous.

Whether or not the two genes studied are indeed evolving faster in *N. inconspicua* lineages than in *Pseudo-nitzschia* and other allogamous Bacillariaceae, three features of our phylogenies seem well supported: (i) *N. inconspicua* is genetically heterogeneous, containing several clearly distinct genotypes (mostly represented by several isolates in our analysis), each with its own unique *rbc*L and LSU genotype; (ii) the genotypes comprise three clades; and (iii) in the *rbc*L tree, *N. inconspicua* is paraphyletic with respect to several other morphologically defined *Nitzschia* species. Thus, in clade A, *N. inconspicua* genotypes 5 and 6, together with *Nitzschia* sp. culture CCMP1532 (sequenced by Hamsher et al. 2011), which conforms

morphologically to *N. inconspicua*, share a most recent common ancestor with *N. frustulum* and *N.* cf. *bulnheimiana*, whereas genotypes 1–4 are paraphyletic with respect to a clade containing *N. amphibia* and *Denticula kuetzingii*. If the gene trees are a faithful record of speciation events – and the agreement between the *rbc*L and LSU trees is encouraging in this respect – then it appears that, for consistency and avoidance of paraphyletic taxa, any classification that continues to recognize *N. amphibia*, *D. kuetzingii, N. frustulum* and *N.* cf. *bulnheimiana* as separate would also have to separate *N. inconspicua* into several cryptic species. This would also agree with the distance-based comparisons made above. However, the number of cryptic species that one might recognize in *N. inconspicua* would be almost arbitrary, since no independent criterion (such as reproductive isolation, in the biological species concept) can be applied in an obligate automict: all lineages evolve separately and so satisfy criteria for recognition as (micro-) species (De Queiroz 2007).

Distribution of genotypes. Most of the *N. inconspicua* sequences used in our analysis came from a relatively small geographical area (Catalonia, Spain) and there was no obvious relationship between the degree of genetic difference and the geographical proximity of the sampling locations. In one case, the same LSU sequence (of G5) was recorded from Spain and California, which agrees with records of widespread LSU genotypes in *N. palea* (one in Spain and Japan; another in Paraguay and Brazil: Trobajo et al. 2009). Where several isolates were made from the same location, they did not always share the same genotype (Table 1), and in two examples, two genotypes were collected from the same locality (syntopic) on the same date (G1_1 and G3_1, from the Lower Ebro River near Ascó; two isolates of G1 and several of G2, from the Set River near Albagés).

Evolution of paedogamy. In terms of the evolution of paedogamy in the *Nitzschia inconspicua* lineages, our gene trees are controversial and it cannot yet be excluded that the results are misleading. For example, assuming that the gene trees are correct in showing that *N. amphibia*, *D. kuetzingii* and some *N. inconspicua* form a clade, it is most likely that the common ancestor of all of these, including the paedogamous *N. inconspicua*, must have been at least facultatively allogamous, since *N. amphibia* and *Denticula* contain allogamous strains (Geitler 1953, 1969). This is because it is implausible that all the mechanisms involved in allogamy (e.g. attraction and recognition of compatible diploid cells during pairing, coordination

of development between paired cells, creation of special structures to facilitate plasmogamy) have been independently evolved in (i) *N. amphibia* and *Denticula* and (ii) other diatoms, including other Bacillariaceae. If the ancestors of the *N. amphibia– D. kuetzingii–N. inconspicua* clade B were allogamous, it is conceivable that the gene trees may not give a reliable estimate of relationships between these taxa, as a result of gene sorting if the sexual ancestor was polymorphic. The reliability of gene-inferred relationships among *N. inconspicua* strains will likewise depend on whether their ancestors were also obligately paedogamous (in which case genetic change and lineage splitting are fully linked and gene trees will be good estimators of relationships), or whether paedogamy has evolved repeatedly from as yet unidentified (and possibly extinct) sexual populations. However, fossil evidence suggests that the clade containing *N. inconspicua* and *N. amphibia* diverged from other *Nitzschia* clades much more than 5 My ago (because diatoms identifiable morphologically as *N. amphibia* and *N. frustulum* are known from Miocene deposits in Hungary and the USA: VanLandingham 1964, 1967, 1991, Hajós 1096), so that there has apparently been ample time for coalescence in the more deeply branching lineages of the *N. inconspicua–N. amphibia–D. kuetzingii* clade.

At a deeper level within the Bacillariaceae, Mann et al. (2013) have shown that it is most likely that paedogamy has evolved independently in the *N. inconspicua* complex and in *N. fonticola*.

Character evolution and use in Bacillariaceae. The gene trees indicate close and sometimes surprising relationships between *N. inconspicua* and several described *Nitzschia* and *Denticula* species. A close relationship between *N. inconspicua* and *N. frustulum* was predictable, since these taxa have already been shown to have similar shape, fibula density and stria density, and to be ultrastructurally identical (Trobajo et al. 2013). In contrast, the close affinity between *N. inconspicua* genotypes 1–4, *D. kuetzingii* and *N. amphibia* is less expected, since these species differ morphologically in several respects from *N. inconspicua* (Table 2). The link between only some of the *N. inconspicua* genotypes and *D. kuetzingii* and *N. amphibia* points to a decoupling of morphological and molecular evolution in the *N. inconspicua* lineages.

Overall, our sequence data indicate that the sect. *Lanceolatae* is not a natural group. Rimet et al. (2011) reached the same conclusion, based on a ML analysis of SSU rDNA, and their tree (ibid., fig. 45) shows the same two clades containing *Lanceolatae* species, one

including *N. inconspicua*, *N. amphibia* and *N. fonticola* with *Pseudo-nitzschia, Fragilariopsis* and *Cylindrotheca*, the other containing *N. palea*, *N. paleaeformis* and *N. communis* as well as species belonging to other *Nitzschia* sections, such as *N. filiformis* (sect. Obtusae) and *N. sigma* (sect. Sigmata). It seems therefore that Grunow's infrageneric classification of *Nitzschia* (in Cleve and Grunow 1880) will need extensive revision.

Furthermore, characteristics often used to separate and classify sect. *Lanceolatae* species, such as the presence or absence of central raphe endings and the structure of the striae in the raphe canal do not define the same groupings as the two genes we analysed here or SSU (Rimet et al. 2011). For example, among the species included in our phylogenies, doubling of the striae within the raphe canal occurs in *N. amphibia*, *N.* cf. *aequorea*, *N. soratensis* and *N. fonticola*, but of these only *N. soratensis* and *N. fonticola* comprise a monophyletic group. Again, species without central raphe endings (e.g. *N. palea*, *N. pusilla*, *N.* cf. *aequorea*, various species of *Pseudo-nitzschia*) are scattered across the trees, consistent with earlier suggestions that the presence/absence of central raphe endings is highly mutable within the fibulate diatoms (Mann 1982). It is also remarkable that a species of a separate genus, *Denticula kuetzingii*, which looks strikingly different from sect. *Lanceolatae* species because of its transversely elongated fibulae, appears to be sister to *N. amphibia* in the *rbc*L tree. These two species also differ with respect to raphe structure, *N. amphibia* having central raphe endings, but agree in one unusual character, namely the presence of cribra as well as hymenes in their areolae (Supplementary Table S5; Mann 1981).

Ecophysiological variation in N. inconspicua. The growth of *N. inconspicua* was studied at salinities between 0.26 ppt and ca 35 ppt and three responses were observed: (i) strains that grew well over the whole salinity range (i.e. euryhaline salinity response); (ii) strains that tolerated all salinities but whose growth declined with decreasing salinity (i.e. almost euryhaline salinity response) and (iii) strains that failed to grow under strictly freshwater conditions (i.e. brackish/marine salinity response). These salinity responses showed no clear correlation with morphology or with the phylogenetic clades found in this study but seem to be genotype specific and related to habitat type. Interestingly, it was the freshwater strains (isolated from habitats with constantly low salinity) that showed the widest tolerance to salinity, while the strains isolated from brackish habitats (not only from those with constant high salinity – e.g. brackish

aquaculture lagoon – but also from environments where salinity fluctuates markedly – e.g. the Ebro Estuary) were the ones that exhibited a narrower tolerance, being unable to grow in freshwater media. Balzano et al. (2011) found that the truly euryhaline strains of *Skeletonema* species were the ones isolated from environments with low or fluctuating salinities, while strains isolated from full or slightly diluted sea water were unable to grow at salinities below 7.5 ppt. These findings and ours seem to support Cox's (1995) suggestion (based on experimental work with clones of *Navicula* species) that marine diatoms may have greater difficulty in adapting their osmoregulation to freshwater than diatoms from freshwater environments do in adapting to sea water. Cox suggested that this would give an extra indicator value to marine/brackish diatoms when found in mixed diatom assemblages. However, it is important to have in mind that there are also 'steno freshwater' species that show very little tolerance to small rises in salinity. An example is *Nitzschia soratensis* (a species that under the LM is very easy to confuse with *N. inconspicua,* see Trobajo et al. 2013), which is unable to grow at salinities above 2.5 ppt (Supplementary Table S6). This underlines the need to avoid confusion in classifying diatoms as "freshwater" or "marine". Do these refer to the salinity of the environment where a species has been recorded or isolated? Or do they refer to the species physiological tolerance to salinity?

Most estuarine diatoms are usually considered to be euryhaline (Guillard and Ryther 1962, Williams 1964, Admiraal 1977, Eppley 1977). However, our work shows that, although all *N. inconspicua* strains studied here grow well at high salinities, only some are truly euryhaline, but that to distinguish these from strains with narrower tolerance (therefore with more indicative value) can only be done with molecular markers. An *in silico* analysis (Supplementary material, Appendix S1) shows that the two barcode markers proposed for diatoms by Mann et al. (2010) and Hamsher et al. (2011) could provide adequate discrimination of the *N. inconspicua* genotypes discovered so far. Ecophysiological and functional diversity among strains that cannot be distinguished morphologically has recently been found also in other diatoms such as *Chaetoceros socialis* (Degerlund et al. 2012, Huseby et al. 2012) and *Pinnularia borealis* (Souffreau et al. 2012). In *C. socialis*, LSU data revealed a clear difference between northern (North Atlantic/Arctic) and Mediterranean strains, which is reflected in growth and photosynthetic responses to temperature but not in morphology, except when the cells form

resting spores. Likewise, in the cosmopolitan terrestrial diatom *P. borealis*, morphologically indistinguishable strains from Antarctica and more temperate localities differ in the optimum and lethal temperatures for growth and are widely separated in gene trees based on *rbc*L and LSU. Our finding of ecophysiological differences in salinity tolerance within *N. inconspicua* has especial practical relevance since it is not only a widespread and abundant diatom species in many aquatic ecosystems, but it is also included in many diatom indices and paleoecological transfer functions (van Dam et al. 1994, Gasse 2002; Rott et al. 2003, Hassan et al. 2009, Hadley et al. 2010, Ryves et al. 2011). Therefore, future studies would be desirable to find out if *N. inconspicua* genotypes also show ecophysiological differences along other environmental gradients such as nutrients, temperature, etc.

Examples such as ours and the *Chaetoceros* and *Pinnularia* studies cited above illustrate that future biomonitoring will need to combine traditional morphological studies with a modern molecular approach involving DNA barcoding (Mann et al. 2010, Hamsher et al. 2011, Zimmermann et al. 2011) and metabarcoding (Stoeck et al. 2009, Hajibabei et al. 2011, Yoccoz 2012). A DNA barcoding approach would be useful not only where ecophysiological different species cannot be distinguished morphologically but also in cases like *N. soratensis* and *N. inconspicua*. Here a major ecophysiological distinction (*N. soratensis* is a steno-freshwater species, Supplementary Table S6) is accompanied by clear ultrastructural and genetic differences, but separating them in the LM is often impractical in ecological and biomonitoring programmes.

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APPENDIX S1

Barcode testing. The usefulness of each potential barcodes suggested by Hamsher et al. (2011) (i.e. *rbc*L-3P and LSU D2–D3) for identifying *N. inconspicua* genotypes was inferred from the presence (or absence) of mutations in the primer binding zone, assuming that if there is no mutation in this zone, this will facilitate primer binding and therefore it will be successfully sequenced. From the whole data set included in our phylogenetic trees, comprising our own and already published GenBank sequences (83 unique *rbc*L and 92 unique LSU sequences), only 1 *rbc*L and 2 LSU sequences showed a mutation in the primer binding zone and none of these were *N. inconspicua* sequences. All *N. inconspicua* genotypes found using the whole *rbc*L and LSU D1/D3 domains were also recovered when only *rbc*L-3P and LSU D2/D3 were considered.

Very similar *N. inconspicua* phylogenetic topology was found using either the whole *rbc*L or the *rbc*L-3P regions, but the topologies obtained for LSU D1/D3 and D2/D3 were more divergent (data not included).

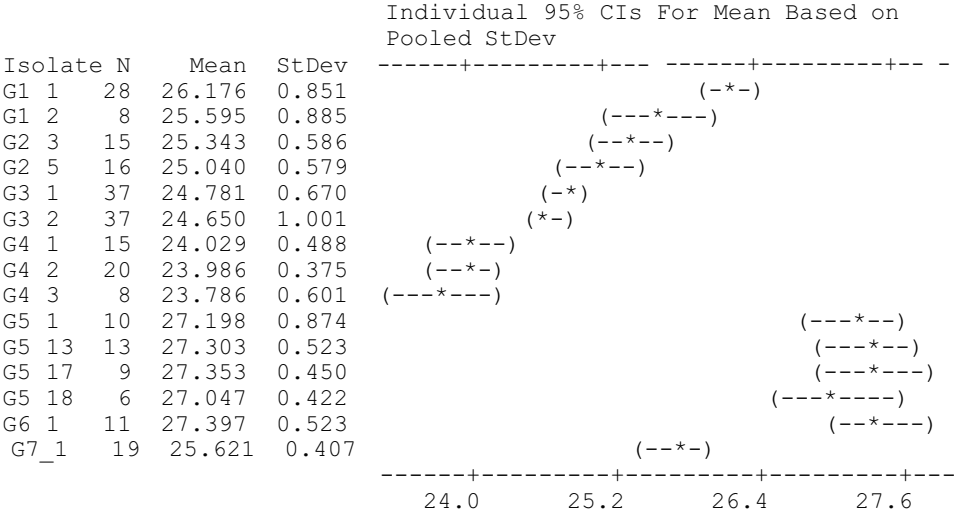
APPENDIX S2

Nitzschia inconspicua

One-way ANOVA: stria density versus isolate with Tukey-Kramer post hoc test

```
Source   DF       SS      MS      F      P
Isolate  14  318.126  22.723  47.24  0.000
Error   237  114.013   0.481
Total   251  432.139

S = 0.6936   R-Sq = 73.62%   R-Sq(adj) = 72.06%
```




```
Pooled StDev = 0.694
```

```
Grouping Information Using Tukey

Method Isolate N    Mean Grouping
G6 1   11  27.3973  A
G5 17   9  27.3533  A
G5 13  13  27.3031  A
G5 1   10  27.1980  A
G5 18   6  27.0467  A B
G1 1   28  26.1761    B C
G7 1   19  25.6205      C D
G1 2    8  25.5950      C D E
G2 3   15  25.3433        D E F
G2 5   16  25.0400        D E F
G3 1   37  24.7808          E F
G3 2   37  24.6497            F G
G4 1   15  24.0287              G H
G4 2   20  23.9865                H
G4_3    8  23.7862              G H

Means that do not share a letter are significantly different.
```

Table S1. List of the 59 new isolates sequenced for this study. Isolate identifier correspond to designation of the strains in Genbank database. Information about the isolation date, voucher slide (all in the diatom collection at the Royal Botanic Garden Edinburgh), habitat and locality coordinates are given.

Taxon	Isolate identifier	Isolation date	Voucher slide	Habitat	Coordinates
N. cf. *aequorea*	DM1004CAT	18/05/2011	E5482	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. cf. ardua	L44	18/03/2010	E5453	Alfacada Bay (Ebro Delta, Spain)	40° 46′ 18.97″ N 0° 46′ 53.80″ E
N. frustulum	Nit24	04/2008	E4553	Es Mercadal Stream (Menorca Island, Spain)	39° 52′ 19.26″ N 4° 8′ 0.48″ E
N. frustulum	Nit25	04/2008	E4554, E5575	Es Mercadal Stream (Menorca Island, Spain)	39° 52′ 19.26″ N4° 8′ 0.48″ E
N. inconspicua	G1_1	18/06/2010	E5568	Lower Ebro River (by Ascó, Spain)	41° 12′ 44.4″ N 00° 33′ 17.8″ E
N. inconspicua	G1_2	18/06/2010	E5455, E5584, E5591	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G1_3	18/06/2010	E5456	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_1	18/06/2010	E5562	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_2	18/06/2010	E5563	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_3	18/06/2010	E5564, E5582, E5590	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_4	18/06/2010	E5565	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_5	18/06/2010	E5566, E5581	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G2_6	18/06/2010	E5567	Set River (by Albagés, Spain)	41° 26′ 53.1″ N 00° 46′ 42.0″ E
N. inconspicua	G3_1	18/06/2010	E5569, E5594	Lower Ebro River (by Ascó, Spain)	41° 12′ 44.4″ N 00° 33′ 17.8″ E

Taxon	Isolate identifier	Isolation date	Voucher slide	Habitat	Coordinates
N. inconspicua	G3_2	18/06/2010	E5458, E5583, E5585, E5588,	Lower Ebro River (by the Xerta assut, Spain)	40° 52′ 58.5″ N 00° 30′ 27.6″ E
N. inconspicua	G3_3	18/06/2010	E5459	Lower Ebro River (by the Xerta assut, Spain)	40° 52′ 58.5″ N 00° 30′ 27.6″ E
N. inconspicua	G3_4	18/06/2010	E5475	Lower Ebro River (by the Xerta assut, Spain)	40° 52′ 58.5″ N 00° 30′ 27.6″ E
N. inconspicua	G4_1	28/10/2009	E5530	Ebro Estuary (Ebro Delta, Spain)	40° 43′ 46.34″ N 0° 52′ 8.45″ E
N. inconspicua	G4_2	28/10/2009	E5531	Ebro Estuary (Ebro Delta, Spain)	40° 43′ 46.34″ N 0° 52′ 8.45″ E
N. inconspicua	G4_3	28/10/2009	E5532, E5586, E5593	Ebro Estuary (Ebro Delta, Spain)	40° 43′ 46.34″ N 0° 52′ 8.45″ E
N. inconspicua	G5_1	18/05/2011	E5480	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. inconspicua	G5_2	18/05/2011	E5483	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. inconspicua	G5_3	18/11/2009	E5533	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_4	18/11/2009	E5534	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_5	18/11/2009	E5535	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_6	18/11/2009	E5536	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_7	18/11/2009	E5537, E5580	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_8	18/11/2009	E5538	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_9	18/11/2009	E5539	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E

Taxon	Isolate identifier	Isolation date	Voucher slide	Habitat	Coordinates
N. inconspicua	G5_11	18/11/2009	E5550	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_12	18/11/2009	E5551	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_13	18/11/2009	E5552	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_14	18/11/2009	E5553, E5579	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_15	18/11/2009	E5554	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_16	18/11/2009	E5555	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_17	18/11/2009	E5556	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_18	18/11/2009	E5557	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G5_19	18/11/2009	E5558	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. inconspicua	G6_1	16.03.2011	E5467	El Clot lagoon (Ebro Delta, Spain)	40°38'58.4"N, 0°41'27.1"E
N. inconspicua	G7_1	17.03.2011	E5465	IRTA aquaculture wastewater lagoon (Ebro Delta, Spain)	40° 37′ 38.88″ N 0° 39′ 39.26″ E
N. palea	L15	09/09/2009	E5540	Lower Ebro River (by the Ginestar Island, Spain)	41° 3′ 4.15″ N 0° 38′ 0.56″ E
N. palea	L16	09/09/2009	E5541	Lower Ebro River (by the Ginestar Island, Spain)	41° 3′ 4.15″ N 0° 38′ 0.56″ E
N. palea	L17	09/09/2009	E5542	Lower Ebro River (by the Ginestar Island, Spain)	41° 3′ 4.15″ N 0° 38′ 0.56″ E
N. palea	L18	09/09/2009	E5543	Lower Ebro River (by the Ginestar Island, Spain)	41° 3′ 4.15″ N 0° 38′ 0.56″ E

Taxon	Isolate identifier	Isolation date	Voucher slide	Habitat	Coordinates
N. palea	L19	09/09/2009	E5544	Lower Ebro River (by the Ginestar Island, Spain)	41° 3′ 4.15″ N 0° 38′ 0.56″ E
N. palea	laia46	-	E5570	Lower Ebro River (by the Mora Island, Spain)	41° 5′ 4.75″ N 0° 39′ 9.12″ E
N. cf. *microcephala*	L56	18/06/2010	E5457	Lower Ebro River (by Flix, Spain)	41° 14′ 25.2″ N 00° 33′ 13.0″ E
N. cf *pusilla*	Nit 44	24.5.2008	E4640-	Threipmuir Reservoir (near Edinburgh, UK)	55° 51′ 27″ N 3° 20′ 17″ W
N. pusilla	L1	11/02/2010	E5527	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. pusilla	L2	11/02/2010	E5528	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. pusilla	L3	11/02/2010	E5529	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. pusilla	L25	11/02/2010	E5549	La Trinitat salt works pond (Ebro Delta, Spain)	40° 35′ 19.65″ N 0° 41′ 21.69″ E
N. soratensis	DM1008MK	29/06/2011	E5524, E5592	Houselop Beck (Durham, UK)	54° 44′ 22.53″ N 1° 51′ 5.80″ W
N. soratensis	DM1009MK	29/06/2011	E5525	Houselop Beck (Durham, UK)	54° 44′ 22.53″ N 1° 51′ 5.80″ W
N. soratensis	NitMK_A1	29/06/2011	E5522	Houselop Beck (Durham, UK)	54° 44′ 22.53″ N 1° 51′ 5.80″ W
N. soratensis	NitMK_A2	29/06/2011	E5523	Houselop Beck (Durham, UK)	54° 44′ 22.53″ N 1° 51′ 5.80″ W
N. soratensis	NitMK_C	29/06/2011	E5526	Houselop Beck (Durham, UK)	54° 44′ 22.53″ N 1° 51′ 5.80″ W
Nitzschia sp.	s0819	4/02/2010	E5598	Trabucador Beach (Ebro Delta, Spain)	40° 38′ 50.15″ N 0° 46′ 12.56″ E
Tryblionella sp.	s0863	2/02/2010	E5597	Buda island (Ebro Delta, Spain)	40° 41′ 60.00″ N 0° 52′ 0.00″ E

Table S2. Genbank accession numbers for sequences downloaded from Genbank and used in phylogenetical analyses. Isolates included within *N. inconspicua* species complex in phylogenetical trees are highlighted in grey.

Taxon	*rbc*L acc.#	LSU acc.#
Bacillaria paxillifera	HQ912491	
Bacillaria paxillifera		AF417678
Cylindrotheca sp.	M59080	
Cylindrotheca closterium	HQ912509	
Cylindrotheca closterium	DQ143045	
Cylindrotheca closterium	DQ143046	
Cylindrotheca closterium	DQ143047	
Cylindrotheca closterium	FJ002104	
Cylindrotheca closterium		EU340870
Cylindrotheca closterium		AF417666
Cylindrotheca fusiformis		AF417665
Denticula kuetzingii	HQ912474	
Fragilariopsis curta		AF417659
Fragilariopsis cylindrus	EF423499	
Fragilariopsis cylindrus	FJ002138	
Fragilariopsis cylindrus		AF417657
Fragilariopsis kerguelensis	EF423501	
Fragilariopsis kerguelensis	EF423500	
Fragilariopsis kerguelensis		AF417658
Fragilariopsis rhombica		AF417656
Fragilariopsis vanheurckii		*AF417660*
Hantzschia amphioxys		AF417677
Hantzschia amphioxys var. *major*	HQ912390	
Neodenticula seminae		GU734794
Neodenticula seminae		GU734797
Neodenticula seminae		GU734796
Neodenticula seminae		GU734793
Neodenticula seminae		GU734795
Nitzschia alba		AF417670
Nitzschia cf. *agnita*		AF417664
Nitzschia cf. *promare*		AF417676
Nitzschia cf. *pusilla*		FJ214163
Nitzschia cf. *pusilla*		GU732414
Nitzschia cf. *pusilla*		FJ214168
Nitzschia cf. *pusilla*		AF417662
Nitzschia cf. *vitrea*		AF417669
Nitzschia communis		AF417661
Nitzschia filiformis	HQ91245	
Nitzschia frustulum		AF417671
Nitzschia inconspicua		AM182195
Nitzschia laevis		AF417673
Nitzschia lecointei		*AF417667*
Nitzschia navis-varingica		AB218885
Nitzschia navis-varingica		AB218886
Nitzschia navis-varingica		AB218887
Nitzschia navis-varingica		AF417675

Taxon	*rbc*L acc.#	LSU acc.#
Nitzschia palea	FN557026	AM183248
Nitzschia palea	FN557027	AM183249
Nitzschia palea	FN557028	AM183250
Nitzschia palea	FN557017	AM183234
Nitzschia palea		AM183238
Nitzschia palea	FN557018	AM183226
Nitzschia palea		AM183227
Nitzschia palea	FN557041	AM183228
Nitzschia palea		AM183229
Nitzschia palea		AM183230
Nitzschia palea	FN557020	AM183232
Nitzschia palea	FN557048	AM183239
Nitzschia palea		AM183247
Nitzschia palea		AM183240
Nitzschia palea	FN557023	AM183246
Nitzschia palea	FN557024	AM183244
Nitzschia palea		AM183241
Nitzschia palea	FN557025	AM183242
Nitzschia pellucida		AF417672
Nitzschia sigmoidea	FN557033	
Nitzschia sp.	EU090049	
Nitzschia sp.	HQ337578	HQ396844
Nitzschia supralitorea		AM182196
Psammodictyon panduriforme	FJ002125	
Pseudo-nitzschia americana	EF423504	EF522108
Pseudo-nitzschia americana		U41390
Pseudo-nitzschia australis		U40850
Pseudo-nitzschia australis		AF417651
Pseudo-nitzschia australis		AM118054
Pseudo-nitzschia brasiliana	FJ150740	
Pseudo-nitzschia brasiliana	FJ150752	
Pseudo-nitzschia brasiliana	FJ150753	
Pseudo-nitzschia brasiliana		AF469673
Pseudo-nitzschia brasiliana		AF469675
Pseudo-nitzschia caciantha	DQ813821	DQ813812
Pseudo-nitzschia calliantha	DQ813825	
Pseudo-nitzschia calliantha		EF566011
Pseudo-nitzschia cf. *subpacifica*		AF417642
Pseudo-nitzschia cf. *hemeii*		AF440777
Pseudo-nitzschia cuspidata	DQ813820	
Pseudo-nitzschia cuspidata		DQ813809
Pseudo-nitzschia delicatissima		EF566016
Pseudo-nitzschia delicatissima		EF566018
Pseudo-nitzschia delicatissima	DQ813819	
Pseudo-nitzschia delicatissima	DQ813823	
Pseudo-nitzschia delicatissima	EF423516	
Pseudo-nitzschia delicatissima	EF520340	
Pseudo-nitzschia delicatissima	EF520341	
Pseudo-nitzschia dolorosa	DQ813822	DQ813813
Pseudo-nitzschia fraudulenta	EF423502	

Taxon	*rbc*L acc.#	LSU acc.#
Pseudo-nitzschia fraudulenta	EF520333	
Pseudo-nitzschia fraudulenta	EF423503	
Pseudo-nitzschia fraudulenta		AF417647
Pseudo-nitzschia galaxiae	EF423515	
Pseudo-nitzschia galaxiae	EF423509	
Pseudo-nitzschia galaxiae	EF423511	
Pseudo-nitzschia galaxiae	EF423512	
Pseudo-nitzschia galaxiae	EF423514	
Pseudo-nitzschia galaxiae	EF423513	
Pseudo-nitzschia galaxiae	EF423510	
Pseudo-nitzschia galaxiae		AY081136
Pseudo-nitzschia galaxiae		AY081137
Pseudo-nitzschia inflatula		AF417639
Pseudo-nitzschia mannii	DQ813824	DQ813814
Pseudo-nitzschia mannii		DQ813816
Pseudo-nitzschia micropora		AF417649
Pseudo-nitzschia multiseries		AF417655
Pseudo-nitzschia multiseries		AF440772
Pseudo-nitzschia multiseries		EU302797
Pseudo-nitzschia multistriata	EF423505	
Pseudo-nitzschia multistriata	EF520337	
Pseudo-nitzschia multistriata	EF520334	
Pseudo-nitzschia multistriata	EF520335	
Pseudo-nitzschia multistriata		AF417654
Pseudo-nitzschia pseudodelicatissima	DQ813817	DQ813808
Pseudo-nitzschia pseudodelicatissima		AF417640
Pseudo-nitzschia pseudodelicatissima		AY550126
Pseudo-nitzschia pseudodelicatissima		AY550127
Pseudo-nitzschia pseudodelicatissima		AY550129
Pseudo-nitzschia pungens	EF423506	
Pseudo-nitzschia pungens	EF423507	
Pseudo-nitzschia pungens		AF417648
Pseudo-nitzschia pungens		AF417650
Pseudo-nitzschia seriata		AF417653
Pseudo-nitzschia seriata		AY452525
Pseudo-nitzschia seriata		AY452526
Pseudo-nitzschia subcurvata		HQ396851
Pseudo-nitzschia subcurvata		HQ396852
Pseudo-nitzschia subcurvata		HQ396845
Pseudo-nitzschia sp.	EF520338	
Pseudo-nitzschia sp.	EF520339	
Pseudo-nitzschia sp. delicatissima	DQ813818	
Pseudo-nitzschia subfraudulenta		AF417646
Pseudo-nitzschia turgiduloides	EF423508	
Tryblionella apiculata	HQ912464	

Table S3. P-distances as % (left-bottom) and bp differences (top-right) within and between *N. inconspicua rbc*L clades (highlighted in grey), and between these clades and some phylogenetically related or morphologically similar *Nitzschia* species. P distances < 1% are highlighted in bold. For the *N. inconspicua* clades, inter-genotype P-distances and bp differences (within brackets) are given along the diagonal.

	Clade A	**CladeB**	**Clade C**	***N. frustulum***	***N.* cf. *bulnheimiana***	***N. amphibia***	***D. kuetzingii***	***N.* cf. *aequorea***	***N. soratensis***
Clade A	3.1–4.7% (22–45)	48–81	48–75	16–42	29–46	58–94	59–99	39–73	106–118
Clade B	5.2–7.2%	**0.1–0.6% (1–8)**	59–60	71–75	61–63	68–70	67–69	77–80	108–109
Clade C	4.6–6.9%	4.5–4.6%	n.a (n.a.)	64	66	71	70	72	102
N. frustulum	1.2–4.3%	5.2–5.5%	4.9%	–	35	83	85	73	107
N.* cf. *bulnheimiana	2.6–4.2%	4.5–4.6%	5.0%	2.6%	–	81	83	72	107
N. amphibia	6.1–8.3%	5.0–5.2%	5.4%	6.1%	6.0%	–	39	92	126
D. kuetzingii	6.3–8.5%	4.9–5.1%	5.3%	6.3%	6.1%	2.9%	–	94	128
N.* cf. *aequorea	5.3–5.6%	5.5–5.9%	5.5%	5.4%	5.3%	6.8%	6.9%	–	99
N. soratensis	7.8–10.9%	8.0%	7.8%	7.9%	7.9%	9.3%	9.4%	7.3%	–

Table S4. P-distances as % (left-bottom) and bp differences (top-right) within and between *N. inconspicua* LSU D1/D3 clades (highlighted in grey), and between these clades and some phylogenetically or morphologically similar *Nitzschia* species. P distances < 1% are highlighted in bold. For the *N. inconspicua* clades, inter-genotype P-distances and bp differences (within brackets) are given along the diagonal.

	Clade A	**Clade B**	**Clade C**	***N.* cf. *bulnheimiana***	***N. amphibia***	***N.* cf. *aequorea***	***N. supralitorea***	***N. soratensis***
Clade A	2.1–3.4% (9–22)	17–40	15–45	32–47	31–53	33	43–45	63–66
Clade B	4.0–6.0%	**0.5–0.9% (3–6)**	20–22	49–51	27–33	44–46	42–47	53–64
Clade C	4.2–9.7%	3.1–4.7%	**0.4% (2)**	50–51	30–37	51–53	42–45	59–62
N.* cf. *bulnheimiana	8.9–14.3%	9.4–10.6%	10.4–10.5%	–	64	52	61	58
N. amphibia	10.1–13.8%	6.4–6.8%	6.3–7.6%	13.2%	–	56	54	58
N.* cf. *aequorea	7.2–7.9%	6.6–7.0%	7.7–10.5%	10.7%	11.5%	–	52	59
N. supralitorea	8.9–10.2%	9.3–9.6%	8.7–9.3%	12.6%	11.1%	10.7%	–	62
N. soratensis	9.1–10.2%	8.5–9.3%	9.1–12.2%	12.0%	12.0%	8.6%	12.8%	–

Table S5. Morphological comparison between members of *Nitzschia inconspicua* clades B and C (genotypes G1–G4), and two related species, *N. amphibia* and *Denticula kuetzingii*. Stria density data for *N. amphibia* and *D. kuetzingii* from Krammer & Lange-Bertalot (1988); other morphological data from this paper, Paddock & Sims (1977), Mann (1978, 1981, and unpublished), Trobajo et al. (2013).

	***N. inconspicua* G1–G4**	***N. amphibia***	***D. kuetzingii***
Stria density (# in 10 µm)	23–31	(13) 14–18 (–20)[1]	13–18[1]
Stria doubling within raphe canal	no	yes, both sides of raphe	sometimes, especially on mantle side of raphe
Striae broken by a plain strip or ridge at the junction between the valve face and distal mantle, with a single small poroid opposite each valve face stria on the distal mantle[1]	no	yes	yes
Subdivision of areolae by a transapical bar	no	no	sometimes
Areola occlusions	hymen	hymen + cribrum	hymen + cribrum
Marginal ridge	absent	absent	present
Fibulae	short, rectangular	short, usually rib-like or double ribs	rib-like, extended across whole width of valve
Central raphe endings	present	present	absent
Pars exterior of valvocopula	plain	warty	warty[1]

[1] Mann, unpublished

Table S6. Growth of four strains of *N. soratensis* (Table 1) after 15 days under different salinity regimes, assessed visually. R1, R2 = Growth assessment of replicates 1 and 2 respectively. +++, good growth; +, very sparse growth; c, very sparse growth and forming chains; –, dead; n.d., no data. The four strains have identical *rbc*L sequences and DM1008MK and DM1009MK also have identical LSU sequences (no LSU data are available for NitMK_C and NitMK_A2). All strains were isolated from Houselop Beck, County Durham, UK.

Strain identifier	Replicate	MBL	96.5% MBL	92.5% MBL	60% MBL	30% MBL	f/2	Salinity response
NitMK_C	R1	+++	+++	c	–	n.d.	n.d.	**Steno freshwater**
	R2	+++	+++	c	–	n.d.	n.d.	
DM1008MK	R1	+++	+++	+	–	n.d.	n.d.	**Steno freshwater**
	R2	+++	+++	+	–	n.d.	n.d.	
DM1009MK	R1	+++	+++	+	–	n.d.	n.d.	**Steno freshwater**
	R2	+++	+++	+	–	n.d.	n.d.	
NitMK_A2	R1	+++	n.d.	n.d.	–	–	–	**Steno freshwater**
	R2	+++	n.d	n.d.	–	–	–	

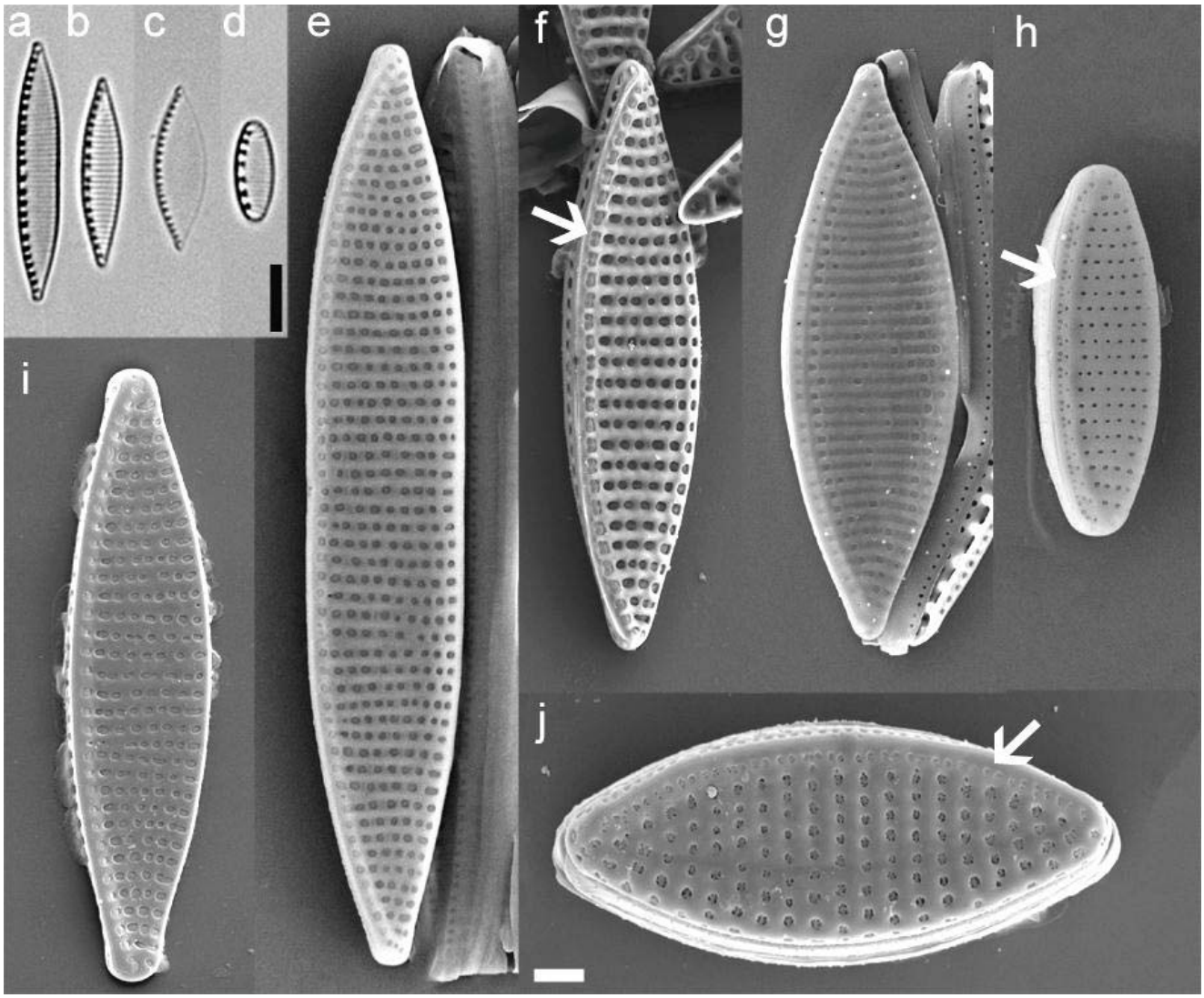


FIGURE S1. *Nitzschia* species phylogenetically related to, or morphologically similar to *N. inconspicua* under LM (a–d) and SEM (e–j). (a, e) *N. frustulum*, (b, f) *N.* cf. *bulnheimiana*, (c, g) *N.* cf. *aequorea*, (d, h) *N. soratensis*, (i) *N. supralitorea*, (j) *N. amphibia*. Note the double areola structure in the raphe channel (arrows) in *N.* cf. *bulnheimiana* (f), *N. soratensis* (h) and *N. amphibia* (j). Scale bars 5 µm for LM micrographs (in d) and 1 µm for SEM micrographs (in j).

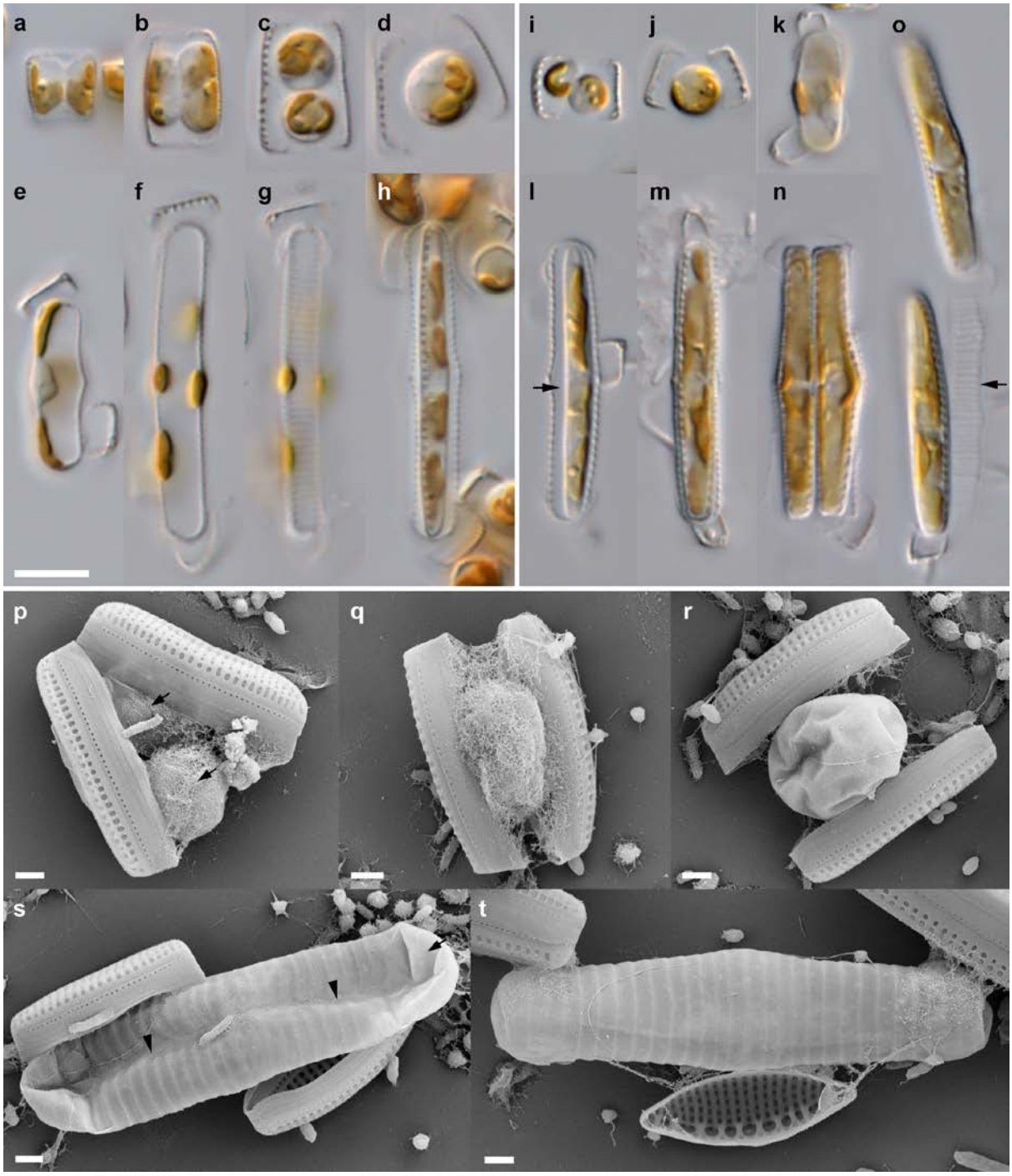


FIGURE S2. Paedogamy in *N. inconspicua* clones: LM (a–o) and SEM (p–t). Scale bars, 5 µm for LM [in (e)], 1 µm for SEM.

(a–h). Clone G2_4, LM. (a) Gametangium immediately after the cytokinetic division at meiosis I: two protoplasts lie side by side within an undehisced cell. (b) Slightly later stage in gametogenesis: the gametangium has dehisced and the gametes are rounding up within it; each gamete contains two chloroplasts. (c) Fully rounded gametes lying one above the other within a dehisced gametangium (the two thecae are clearly separated. This arrangement is commonly observed in *N. inconspicua*, but sometimes the gametes do not become rearranged within the gametangium, as in (i). (d) Zygote. (e) Partly expanded

auxospore. (f, g) Two focuses of a fully expanded auxospore: one of the gametangial thecae is associated with each pole. (h). Fully formed initial cell within the auxospore; it contains four chloroplasts arranged in a line.

(i–o) Clone L65, LM. (i) Gametes side by side within a dehisced gametangium. (j) Zygote. (k) Early stage in auxospore expansion. (l) Fully expanded auxospore containing initial cell: There is a wide separation (arrow) between the perizonium and initial cell, so that the perizonium does not directly control the shape of the enlarged cells, though it does control the length. (m) Fully formed initial cell in girdle view, now expanded to fill the perizonium. (n) Divided initial cell, still within perizonium. (o) Escape of the two daughter cells of an initial cell, leaving behind the perizonium (arrow) as a husk.

(p–t) Clone G4_2, SEM, critical-point dried material. The small rod-shaped and spherical cells are bacteria. (p) Two gametes (arrows) surrounded by fibrillar material (probably dehydrated mucilage) within a dehisced gametangium [cf. (c)]. (q) Young zygote, with abundant fibrillar material. (r) Mature zygote, surrounded by a robust wall (the incunabula) containing silica scales (their presence was established by acid cleaning). (s) Expanded auxospore, before formation of the initial valves. The incunabula have persisted as caps (e.g. arrow) over the ends of the auxospore; between them is a perizonium, containing a series of bands, all of which (except the central band) open along a ‘suture’ (arrowheads) along one side. The gametangial thecae flank the auxospore. (t) Expanded auxospore containing initial valves (which have prevented collapse): this cell is seen from the side opposite the suture. Fibrillar material derived from the gametes and zygote persists on the caps over the ends of the auxospore.

General discussion

The present discussion aims to give an overall synthesis of the results found in each of the previous chapters. One of the main outcomes of the thesis is that the benthic diatom community can potentially be used as a bioindicator of the main anthropogenic impacts in the Ebro Estuary (and, possibly, in other salt-wedge estuaries). However, several obstacles need to be solved prior its practical use in future biomonitoring strategies, not only as a result of a poor performance of existing diatom indices, but also due to the lack of ecological and taxonomical knowledge of diatom species inhabiting these estuarine ecosystems.

Estuarine hydrology, salinity and diatom ecology

The Ebro Estuary, like other estuarine ecosystems previously studied (reviewed in Trobajo & Sullivan 2010), comprises a well-established benthic diatom community both at the superficial and at deep levels within the water column. However, neither diatoms nor other components of phytobenthos are recommended as aquatic flora quality elements for any type of transitional and coastal waters under the Water Framework Directive (WFD). The exclusion of phytobenthos in open coastal waters is reasonable since it is not present at deep water levels. In contrast, in the case of inner and relatively shallow transitional water bodies such as coastal lagoons and estuaries, where the bottom sediments are close enough to the surface to receive adequate light for photosynthesis, the phytobentos is often present, as it is the case of the Ebro Estuary.

The ecological status of the Ebro River has been assessed by the Hydrographical Confederation of the Ebro (CHE, http://www.chebro.es/) using the benthic diatom community. Sampling sites covered most of the basin, including three sites situated 30 - 40 km upriver from the sea, which enclose the upper limit of the Ebro Estuary. In this area, the ecological status of the Ebro River in 2007 - 2008 (last period sampled by the CHE) was classified as “moderate” using IPS and CEE and “poor” using IBD diatom indices. Despite these few cases, the Ebro Estuary has not been monitored by the CHE, even though the estuary and the river are tightly connected in their ecological status and the physicochemical and hydrological characteristics of the Ebro Estuary depend strongly on the influence of upstream processes.

This thesis has shown that the benthic diatom community of the Ebro Estuary is mainly influenced by estuarine hydrological dynamics (i.e. marine influence and long periods of low and stable flows that determine the position and persistence of the salt wedge). The hydrology of salt-wedge estuaries is influenced by the balance between the river and the sea and encompasses the interaction of their associated environmental fluctuations. Specifically, the present thesis has mostly focused on salinity, since it is one of the key gradients that characterise estuaries (Hansen & Rattray 1966) and it has been previously regarded as an important factor driving estuarine diatom community composition (e.g. Bak et al. 2001; Laird & Edgar 1992; Thornton et al. 2002; Watt 1998). Salinity affects the growth

of diatom species inhabiting the Ebro Estuary, though the magnitude of the response and salinity preferences and tolerances are species-specific.

Benthic diatom species inhabiting estuaries have been previously documented as being euryhaline and having wide ecological ranges (Bate & Smailes 2008; Forster et al. 2006; Hassan et al. 2006; Sullivan & Currin 2000; Underwood 1994; Underwood & Kromkamp 1999; Wilderman 1987). These studies have been carried out in tidal estuaries where being euryhaline is an advantage, since there is an oscillatory salinity gradient due to the range and frequency of tides. In contrast, the water column in salt-wedge estuaries can be highly stratified into freshwater or marine conditions for long periods. In the Ebro Estuary, euryhaline diatom species will dominate during fluctuating conditions, e.g. under situations of a strong variable flow, where there is a retreat (high flows) or a rapid intrusion (low flows) of the salt wedge, especially in those sites closer to the sea. However, under stable hydrological conditions (e.g. a highly stratified water column during low flows or a fluvial situation under long periods of high flows), several species with a narrower salinity spectrum can coexist with euryhaline species in high abundances along the vertical and horizontal scales.

The presence of diatom species described from marine environments such as *Amphora* aff. *luciae, Cocconeis* cf. *neothumensis* var. *marina, Gomphonemopsis obscura, Parlibellus* cf. *berkeleyi and Planothidium iberense* in the Ebro Estuary will be especially significant due to their role as indicators of a long-term salt-wedge presence as a consequence of a persistent low river flow. Although these species showed high salinity optima (> 17 mS/cm) and high specificities for estuarine conditions (> 96%), they were found both at deep and superficial water levels in those sites close to the sea, showing a tolerance to salinity fluctuations. We can conclude that most benthic diatom species in the Ebro Estuary are tolerant to environmental fluctuations, although the degree of tolerance is species-specific. As a result, the identification of groups of indicator diatom species rather than their individual abundances will be desirable to properly infer the environmental conditions present in the estuary.

Although salinity can be considered a surrogate of marine influence and salt-wedge dynamics, multivariate analyses and incongruity between field and laboratory salinity preferences for some species suggest that the diatom community in the Ebro Estuary is affected by multiple natural and anthropogenic stressors. The intrinsic physicochemical co-variation in estuaries between salinity and other factors (e.g. nutrients, oxygen, temperature) and the effect of others parameters that are difficult to identify and measure in the field (e.g. habitat competence, colonisation, grazing) make it very difficult to determine the separate effects of single variables (Cox 1993; Trobajo et al. 2004b; Underwood et al. 1998; Underwood & Provot 2000). Moreover, the effect of natural and anthropogenic stressors in aquatic biota inhabiting strongly human-influenced estuaries such as the Ebro Estuary can be similar, which can compromise the evaluation of their ecological status (Elliott & Quintino 2007; Puente & Díaz 2008). In that sense, experimental studies can help to disentangle the effects of relevant stressors on the benthic diatom community of the Ebro Estuary. Further experimental studies considering the response of diatom species to salinity combined with other

environmental gradients related to marine influence and salt-wedge dynamics (e.g. nutrient and flow fluctuation, oxygen depletion, etc.) would be interesting.

The use of benthic diatoms as bioindicators in the Ebro Estuary

The benthic diatom community is a potential bioindicator of the altered conditions in the Ebro Estuary, since it was found to be sensitive to the current main anthropogenic pressure, i.e. hydrological alteration of the lower Ebro River flow and the salt-wedge dynamics. As a consequence, different groups of diatom species were found to be indicative of the two main ecological situations of the Ebro Estuary: i.e. riverine conditions (freshwater) and estuarine conditions (brackish and marine). The presence of indicator species of estuarine conditions together with the indicator species of a long-term persistent salt wedge can be used in future studies as a sign of anthropogenic pressure during specific periods, such as in spring (when high flows should prevail), or when they colonise further upstream than the most frequent limit of the salt wedge (18 km from the estuary mouth, near Gràcia Island).

The use of benthic diatoms as bioindicators in the Ebro Estuary showed several methodological advantages over other bioindicators that are currently considered as biological quality elements in transitional systems by the WFD (Annex V). For instance, the benthic diatom community in highly dynamic ecosystems such as the Ebro Estuary is mainly affected by several environmental stressors that likely override the effect of substratum type, as has been previously reported for freshwater and coastal ecosystems (Potapova & Charles 2005; Lane et al. 2003; Snoeijs 1994; Winter & Dutie 2000). Therefore, a diatom community inhabiting artificial substrata (i.e. fired-clay bricks) may be considered representative of a natural community of the Ebro Estuary, facilitating biomonitoring strategies. Cobbles and boulders are the recommended substrata for sampling diatoms under the WFD procedures (Kelly et al. 1998). Unfortunately, the access to this type of substratum in the Ebro Estuary is very limited, since the water column is several meters deep and the sediment is mainly composed of sand (Nebra et al. 2011). The use of artificial substrata will also minimise the effect of sampling different substratum types within the water column and will also allow controlled sampling conditions and constant exposure times, which are regarded as significant factors affecting phytobenthos composition and therefore, diatom-based indices values (Anderson 1995, 1999; Biggs 1988; Kelly et al. 1998).

Advective circulation becomes more significant under long periods of a well-established salt wedge, which will be mainly reflected in an increase of salinity concentration at superficial level as the estuary approaches to its mouth. Once the superficial waters have reached conductivities of approximately 3 mS/cm or more, most estuarine diatom species can inhabit both deep and superficial water layers. Therefore, we can identify human altered conditions that involve an increase of salinity levels by monitoring the diatom community colonising the superficial water layer. Sampling at superficial levels is an advantage for biomonitoring procedures in relatively large and deep estuaries such as the Ebro Estuary, where the strong flow fluctuation and flood events can impede the recovery of artificial substrata at deep water levels.

Difficulties in the use of benthic diatoms to evaluate ecological status of the Ebro Estuary

Although benthic diatoms have proved to be potential bioindicators of altered hydrological conditions in the Ebro Estuary, the use of existing diatom-based indices to assess the ecological status of salt-wedge estuaries (and other transitional waters) is not recommended. This is because most diatom indices were developed from and for freshwater ecosystems of North and Central Europe (Kelly et al. 2009) and several indicator species from the Ebro Estuary are not included in their database, especially those under estuarine conditions, where > 60% of the indicator species were not considered in most indices. Indeed, some of the species found during this study appear to be new to science (and one has been described formally as *Planothidium iberense* Rovira & Witkowski), while others are well documented within brackish-marine environments such a*s Achnanthes amoena*, *Amphora polita, Navicula mollis*, *Nitzschia constricta* or *N. prolongata* (Chang 1992; Krammer & Lange-Bertalot 1988, 1991; Levkov 2009; Witkowski et al. 2000).

Existing diatom indices were originally designed to assess the nutrient and organic pollution of water bodies as a consequence of the Urban Wastewater Treatment Directive (European Community, 1991). Although the WFD introduced the evaluation of the "ecological status" as a holistic approach that considers not only the water quality but also the structure and function of the ecosystem in response to anthropogenic pressures (Kelly 2011), no adjustments or specific indices have been developed for this purpose. Since "ecological status" includes the response of the ecosystem to several types of pollution and other impacts, an extra level of caution has to be taken when applying nutrient-based indices to ecosystems that are affected by other types of contamination (e.g. heavy metals, acidity, halogenated hydrocarbons) or other anthropogenic pressures such as hydrological alteration. In the Ebro Estuary, ecological status values resulting from the application of existing indices were strongly influenced by the salinity gradient and very weakly correlated to the nutrient levels. Only the trophic diatom indices (TDI and TID) showed a consistent negative correlation with nutrients, although the resulting low water class (poor, bad) do not reflect the trophic status of the lower Ebro River when phosphorus concentrations are considered, which would be classified as mesotrophic according to Dodds et al. (1998) classification of temperate rivers.

Existing diatom indices have the assumption that the increase of species tolerant to environmental fluctuations will be related to a significant nutrient-related disturbance as a consequence of human activities. Although the results of the present thesis agree with the expected dominance of eutraphentic and α or β-mesosaprobous diatom species in transitional waters (Trobajo et al. 2004b, Witkowski et al. 2009), their distributional patterns can also be indicative of a high tolerance to other environmental disturbances regardless of nutrient and/or organic matter levels. Moreover, high abundances of these species do not always imply a decrease in ecological status, as stated by the "Estuarine Quality Paradox" (Dauvin & Ruellet 2009; Elliot & Quintino 2007). In the Ebro Estuary, results suggest that the high abundance of stress-tolerant species under estuarine conditions (and the consequent low ecological status assessment through application of existing indices) is mostly related to fluctuating conditions caused by the salt-wedge dynamics, which do not necessarily

constitute altered conditions. Although nutrient levels in the Ebro Estuary follow a spatial and temporal gradient, their present relatively low values mean that nutrients exert a much weaker pressure on benthic diatom communities than the hydrological alteration of the Ebro River flow and its salt-wedge persistence.

Autoecological data for most diatom species considered in existing indices have been obtained from freshwater ecosystems and therefore their ecological preferences under brackish-marine situations (if any) is unknown. Regarding to salinity, diatom species described from “freshwater” or “marine” habitats can be either euryhaline, tolerant to salinity at diferent levels or stenohaline. Salinity preferences for several diatom species in the Ebro Estuary did not always agree with previous work in other similar ecosystems, nor was there agreement between our field data and laboratory experiments. For example, *N. inconspicua* conductivity optima in our field studies was 4 mS/cm (~ 2.4 ppt at 20ºC), while in the laboratory experiments all clones of this species grew well from salinities of 14 ppt up to 35 ppt. In other studies, its salinity optima have been found at 0.4 mS/cm in U.S.A. rivers (Potapova & Charles 2003) and at 0.9 mS/cm in the oligohaline reaches of other estuaries (Licursi et al. 2010); and it has been described as a fresh-brackish diatom species (e.g. Van Dam et al. 1994) characteristic of brackish situations in other transitional and coastal waters such as the Baltic Sea (Ulanova & Snoeijs 2006; Ulanova 2009). The determination of ecological ranges from distributional patterns can be highly biased depending on the particular physico-chemical characteristics of the considered environment. The present thesis showed that experimental studies under controlled conditions are crucial to elucidate the potential ecological ranges of diatoms, especially needed for those species that are considered in most diatom-based indices. Although this can be a long process, it is necessary to determine the species-specific responses to the particular environmental gradients that the biological indices are designed to evaluate.

The lower Ebro River and its estuary have been under intense industrial and agricultural influences for decades (Ibàñez et al. 2012). Since the present thesis is the first study on the benthic diatom community of the Ebro Estuary, it is not possible to discern how degraded the actual diatom community is when compared to reference conditions (i.e. before human activities). Moreover, the specific geomorphological, physicochemical and climatic characteristics of the Ebro Estuary make it difficult to find similar ecosystems which can be considered as reference conditions. Therefore, future biomonitoring strategies in the Ebro Estuary should involve an interdisciplinary approach combining modelling with field surveys and experimental studies, in order to generate sound hypotheses on reference conditions and the specific response of benthic diatom community to potential human stressors (Muñoz et al. 2012).

Hidden diversity in diatoms and the importance of a multidisciplinary approach

Most diatom species in the Ebro Estuary are small (< 30 µm) and some of them (e.g. *E. minima*, *N. inconspicua*, *N. filiformis*, *N. palea* and *N. pusilla*) show a variability in the classic morphological characters (i.e. length, width, stria and fibula density) due to their life cycle and/or as a response to salinity. Several of these species have been largely confused in traditional flora works, generally due

to insufficient taxon discrimination. If a diatom species cannot be properly identified, its ecological preferences will not be accurately described and this will affect its indicator value and the assessment of ecological status of any ecosystem using indices based on its autoecological data. Therefore, any development of a diatom-based biomonitoring strategy will be hampered by a lack of a sound taxonomy.

The present thesis studied the ecologically important *N. inconspicua* from a multidisciplinary approach (a combination of detailed morphological, molecular, ecophysiological and reproductive analyses) in order to refine its taxonomy and ecological preferences regarding to salinity. *N. inconspicua* has been often misidentified and/or combined with *N. frustulum* and its ranges of size, shape, fibula and stria density often overlap with other *Nitzschia* species such as *N. abbreviata*, *N. costei*, *N. epiphytica*, *N. innominata*, *N. invisitata* and *N. soratensis* (e.g. Hoffmann et al. 2011; Krammer & Lange-Bertalot 1988; Lange-Bertalot 1977, 1993; Lange-Bertalot & Simonsen 1978; Trobajo et al. 2004a, b; this thesis). In Chapters 1, 2 and 3 of this thesis and according to the available published taxonomic literature, the longer valves (15 - 30 µm aprox. in this thesis) with slightly protracted ends were identified as *N. frustulum* while the shorter ones (5 - 15 µm aprox. in this thesis) with more rounded ends were attributed to *N. inconspicua*. However, later in the thesis, both types of valve were found to be produced within each of several *N. inconspicua* monoclonal cultures depending on its life-cycle stage. Length is a highly variable morphological character in diatoms. Length alters during the life cycle as a natural consequence of the method of cell division and can also be influenced by environmental conditions, e.g. through alteration in the rate of cell division or alteration in the amount of change per cell division (e.g. Amato et al. 2005; Håkansson & Chepurnov 1999; Potapova & Snoeijs 1997; Trobajo et al. 2004a; 2006), or through induction of abrupt size reduction (Chepurnov et a. 2004). This thesis has concluded that it cannot be used any longer as a taxonomic criterion to distinguish *N. frustulum* and *N. inconspicua.*

In Chapter 4, detailed study of frustule morphology allowed discrimination between *N. inconspicua* and morphologically similar species such as *N. frustulum*, *N. invisitata* and *N. soratensis*. *N. frustulum* has wider valves (> 3 µm) than *N. inconspicua* (< 3 µm) and, therefore, the valves misidentified as *N. frustulum* in Chapters 1, 2 and 3 belong to long cells of *N. inconspicua* after auxosporulation. *N. inconspicua* and *N. soratensis* can not only be differentiated by the different apices and fibula shape as well as by the ultrastructure of the striae, but they also show a very distant phylogenetic relationship and have different ecophysiological responses to salinity, and therefore the effort required for discriminating them is essential in future ecological works. However, Chapter 5 evidenced that morphological characters were insufficient to identify genotypic or phenotypic variability found within different populations of *N. inconspicua*. With the exception of maximum length observed, none of the other “classical” morphological characters (i.e. width, striae and fibulae) strongly varied between *N. inconspicua* isolates that showed a distinct genetic, reproductive and/or ecophysiological pattern.

N. inconspicua contained a high genetic diversity for *rbc*L and LSU when compared with other *Nitzschia* species, such as *N. palea* or *Pseudo-nitzschia* species (Trobajo et al. 2009, 2010). The populations of *N. inconspicua* studied also showed different salinity responses suggesting an ecophysiological diversity within the species complex. Moreover, *N. inconspicua* populations also showed slight differences in its size restitution pattern, most of them with auxosporulation through an automictic process named paedogamy, but a few showing a vegetative enlargement. The present thesis suggests that the euryhaline behaviour of this species complex could be explained by an overlap of several cryptic species with distinct salinity preferences. However, the use of a species concept to establish intraspecific limits in *N. inconspicua* is not straightforward. While the phylogenetic species concept shows that *N. inconspicua* has a paraphyletic origin and therefore cannot be treated as a single species, the morphological species concept gives very little evidence about species boundaries and the biological species concept is inapplicable since *N. inconspicua* is an automictic diatom species.

The discovery of an ecophysiological diversity within *N. inconspicua* can be the first step to refine the broad ecological ranges and indicator values previously described for this diatom species (e.g. Van Dam et al. 1994). Future studies would be desirable to investigate whether this ecological variability extends to other environmental gradients, such as nutrients, temperature or oxygen. The fact that salinity preferences are genotypically specific and not related to morphology suggests the ecophysiological diversity within *N. inconspicua* can only be detectable through molecular markers (e.g. DNA barcodes), as has been found in other diatom species complexes such as *Chaetocheros socialis* (Degerlund et al. 2012; Huseby et al. 2012) and *Pinnularia borealis* (Souffreau et al. 2012). The discovery of ecophysiological diversity within *N. inconspicua* is especially relevant since not only is an abundant and frequent diatom species in the phytoplankton (Pérez et al. 2009) and phytobenthos of the Ebro Estuary (this thesis), but it is also a widespread species in most aquatic ecosystems and is included in most diatom indices and palaeoecological transfer functions (Gasse 2002; Hadley et al. 2010; Hassan et al. 2009; Rott et al. 2003; Ryves et al. 2011; van Dam et al. 1994).

In conclusion, multidisciplinary approaches (combining morphological, molecular, ecophysiological and reproductive data) seem to be the only way to properly assess diatom diversity (at all levels of organisation) and are essential to understand what this hidden diversity will implicate in future biomonitoring studies not only in the Ebro Estuary, but also in other transitional waters.

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Conclusions

1. In the Ebro Estuary, as a consequence of the salt-wedge dynamics, two contrasting environmental conditions were observed: a riverine condition with no or very weak marine influence and; an estuarine condition under marine influence at the deep water level but also (at a minor scale) at the superficial level in sites closer to the sea.

2. There is a well-established benthic diatom community in the Ebro Estuary at superficial and deep water levels, both for riverine and estuarine conditions. Most of the 160 species found are cosmopolitan species that have been widely documented in other environments, such as *Cocconeis placentula* var. *euglypta* (found in the 99% of the samples), although several species (e.g. *N. inconspicua*) have a confused taxonomy and their identification has not been straightforward.

3. *Planothidium iberense* was described as new diatom species to science, and it can be considered as indicator of a particular ecological condition in the Ebro Estuary.

4. The hydrological dynamics of the Ebro Estuary was the main factor affecting benthic diatom community both at spatial and temporal scales. Marine influence appeared as the principal factor affecting the spatial distribution and composition of diatom species. The temporal variability in the diatom community was associated to the Ebro River flow fluctuations and long periods of low flow.

5. The response of benthic diatom community to hydrological dynamics of the Ebro Estuary was stronger than the single effect of salinity, substratum type or nutrient concentration. Both marine influence and the hydrological regime of Ebro River flows are complex environmental processes that comprise the interaction of several physicochemical factors. The separate effects of these factors cannot be disentangled from field studies.

6. Although most benthic diatom species in the Ebro Estuary are tolerant to environmental fluctuations, it was possible to identify groups of diatom species indicators of riverine and estuarine conditions. Moreover, several species with low indicator values, but present only under specific conditions, allowed the identification of situations of a long term well-established salt wedge.

7. *Achnanthidium minutissimum*, *Amphora pediculus*, *A.* cf. *vetula*, *Cocconeis placentula* var. *euglypta*, *C. placentula* var. *trilineata*, *Navicula antonii*, *N. cryptotenella*, *N.* cf. *cryptotenelloides* and *Nitzschia amphibia* are indicator diatom species of riverine conditions.

8. *Amphora polita*, *Navicula gregaria*, *N.* aff. *mollis*, *N. perminuta*, *N. recens*, *Nitzschia constricta*, *N. inconspicua* (misidentified and referred to as *N. frustulum*) and *Tabularia fasciculata* are indicator diatom species of estuarine conditions.

9. *Amphora* aff. *luciae*, *Cocconeis* cf. *neothumensis* var. *marina*, *Diploneis* sp., *Gomphonemopsis obscura*, *Parlibellus* cf. *berkeleyi* and *Planothidium iberense* are indicator diatom species of a long term well-established salt wedge.

10. Nowadays, the main anthropogenic pressure in the Ebro Estuary is the increased salt-wedge presence and flow stability as a result of a highly regulated lower Ebro River flow, due to the agricultural activities and reservoirs functioning since the 1960s.

11. Benthic diatom communities are potential biological indicators of anthropogenic pressures in the Ebro Estuary, since altered hydrological conditions were detectable through the presence of several indicator diatom species of estuarine and long term well-established salt-wedge situations.

12. The existing diatom indices did not properly assess the ecological status of the Ebro Estuary since they were not properly correlated with nutrients and several indicator diatom species of estuarine conditions were not included in their database. Therefore, their effectiveness in transitional waters has to be tested before their application.

13. Salinity affected the growth and valve morphology of the diatoms species studied; *Eolimna subminuscula, Nitzschia filiformis* var. *conferta, N. inconspicua* (misidentified and referred to as *N. frustulum*), *N. palea* and *N. pusilla*, although the response was different for each species.

14. The effects of salinity on the morphology of the five diatom species studied were very small and, therefore, do not undermine the usefulness of some classical taxonomic characters (i.e. width and stria density) for the discrimination of these species in environments with strong salinity fluctuations.

15. Four of the five diatom species studied showed good or moderately good growth in all the salinity treatments, which agrees with their broad distribution in the Ebro Estuary. However, cultures isolated from brackish environments (i.e. *N. inconspicua* and *N. pusilla*) grew better under low salinities than cultures isolated from freshwater environments (i.e. *N. filiformis* var. *conferta* and *N. palea*) under high salinities.

16. *N. frustulum, N. inconspicua, N. soratensis and N. invisitata* are taxonomically distinct independent species that with careful examination can be distinguished under LM. *N. inconspicua* and *N. frustulum* have different widths (averages of < 3 μm in *N. inconspicua* and > 3 μm in *N. frustulum*). *N. invisitata* and *N. soratensis* not only can be distinguished from *N. inconspicua* by their different ultrastructure, but also by their “torn notebook” fibulae shape. Moreover, *N. invisitata* has coarser areolae, slightly lower stria density and generally more

distantly spaced median fibulae, and *N. soratensis* has more rounded apices and less conspicuous striae. Finally, *N. soratensis* and *N. inconspicua* had a distant phylogenetic relationship for *rbc*L and LSU, and whilst the former was found to grow under strictly freshwater conditions, the latter grew from freshwater to salinities up to 35 ppt.

17. *N. frustulum* var. *subsalina* and *N. boliviana* are synonyms of *N. inconspicua*. The three species show the same ultrastructural features and a complete overlap in metrics observed.

18. *N. inconspicua* is paraphyletic with respect to other *Nitzschia* and *Denticula* species and comprised a high genetic diversity for *rbc*L and LSU, with several genotypes grouped in three clades. Moreover, isolates with different genotypes showed differences in the size restitution pattern and in the growth as a response to the salinity treatments.

19. The ecophysiological variability within *N. inconspicua* is only detectable through molecular techniques, such as the DNA barcoding. Though there were statistically significant differences in morphological characters between populations, they were subtle, often overlapped and did not indicate the different responses to salinity observed under experimental studies.

20. The ecophysiological and genetic variability within *N. inconspicua* isolates seem to indicate that the broad environmental tolerances attributed to the species complex could enclose several morphologically undistinguishable species with distinct ecophysiologies (at least for salinity).

Benthic diatom community of the Ebro Estuary

List of diatom taxa and relative abundances

List of the 160 diatom taxa encountered in the Ebro Estuary (ranged alphabetically) with their authorities and their relative abundances (RA) considering all samples. Plates are also indicated for those taxa illustrated in this thesis. LM: light microscope, SEM: scanning electron microscope.

Diatom taxa	RA (%)	Plates
Achnanhtes amoena Hustedt	0.796	1 (LM)
Achnanthes brevipes C. Agardh	0.069	
Achnanthes hungarica (Grunow) Grunow	0.001	
Achnanthes lanceolata (Brébisson ex Kützing) Grunow	0.039	
Achnanthes longipes C.Agardh	0.001	
Achnanthes punctata Round	0.001	
Achnantes sp.	0.278	1 (LM)
Achnanthidium subatomus (Hustedt) Lange-Bertalot	0.001	
Achnantidium minutissimum (Kützing) Czarnecki	1.419	1 (LM), 6 (SEM)
Amphora aequalis Krammer	0.029	
Amphora helenensis Giffen	0.146	
Amphora inariensis Krammer	0.287	1 (LM), 6 (SEM)
Amphora indistincta Levkov	2.222	1 (LM), 6 (SEM)
Amphora lineata Gregory	0.001	
Amphora aff. *luciae* Cholnoky	0.493	1 (LM), 6 (SEM)
Amphora aff. *margalefii* X.Tomàs	0.022	
Amphora cf. *meridionalis* Levkov	0.611	1 (LM), 6 (SEM)
Amphora ovalis (Kützing) Kützing	0.212	1 (LM)
Amphora pediculus (Kützing) Grunow	8.324	1 (LM), 6 (SEM)
Amphora polita Krasske	0.584	1 (LM), 6 (SEM)
Amphora cf. *vetula* Levkov	1.828	1 (LM), 6 (SEM)
Bacillaria paxillifera (O.F. Müller) Hendey	2.567	1 (LM)
Berkeleya rutilans (Trentepohl ex Roth) Grunow	0.097	
Caloneis bacillum (Grunow) Cleve	0.002	
Cocconeis cf. *neothumensis* var. *marina* De Stefano, Marino & Mazzella	0.705	2 (LM), 7 (SEM)
Cocconeis pediculus Ehrenberg	1.031	2 (LM), 7 (SEM)
Cocconeis placentula var. *euglypta* (Ehrenberg) Grunow	14.62	2 (LM), 8 (SEM)
Cocconeis placentula var. *placentula* Ehrenberg	1.343	2 (LM), 8 (SEM)
Cocconeis placentula var. *trilineata* (M. Peragallo & J. Héribaud) Cleve	5.305	2 (LM), 8 (SEM)
Cocconeis scutellum Ehrenberg	0.026	
Cocconeis stauroneiformis (W. Smith) Okuno	0.064	
Cocconeis sp.	0.145	

Diatom taxa	RA (%)	Plates
Cyclostephanos invisitatus (Hohn & Hellermann) Theriot, Stoermer & Håkasson	0.024	
Cyclotella atomus Hustedt	0.082	
Cyclotella meneghiniana Kützing	0.256	2 (LM), 9 (SEM)
Cyclotella ocellata Pantocsek	0.019	
Cyclotella radiosa (Grunow) Lemmermann	0.001	
Cyclotella stelligera (Cleve & Grunow) Van Heurck	0.001	
Cymatopleura solea (Brébisson) W.Smith	0.001	
Cymbella lanceolata (C.Agardh) Kirchner	0.010	
Cymbella prostata (Berkeley) Cleve	0.012	
Cymbella sp.	0.189	
Cymbella tumida (Brébisson) van Heurck	0.064	
Denticula crotonensis	0.326	
Denticula sundayensis Archibald	0.003	
Diadesmis confervacea Kützing	0.116	
Diatoma moniliformis Kützing	0.048	
Diatoma tenuis C.Agardh	0.025	
Diatoma vulgaris Bory de Saint-Vincent	0.145	
Diploneis aff. *bombus* (Ehrenberg) Ehrenberg	0.078	
Diploneis cf. *smithii* (Brébisson) Cleve	0.013	
Diploneis sp.	1.148	2 (LM), 9 (SEM)
Eolimna minima (Grunow) Lange-Bertalot & W.Schiller	0.199	
*Eolimna subminuscula (*Manguin) Moser, Lange-Bertalot & Metzeltin	0.435	3 (LM), 10 (SEM)
Fallacia clepsidroides Witkowski	0.406	2 (LM), 9 (SEM)
Fallacia subhamulata (Grunow) D.G.Mann	0.034	
Fallacia tenera (Hustedt) D.G.Mann	0.183	
Fragilaria capucina Desmazières	0.001	
Fragilaria geocollegarum Witkowski & Lange-Bertalot	0.018	
Fragilaria improbula Witkowski & Lange-Bertalot	0.012	
*Fragilaria pulchella (*Ralfs ex Kützing) Lange-Bertalot	0.067	
Fragilaria sopotensis Witkowski & Lange-Bertalot	0.055	
Gomphoneis clevei (Fricke) Gil	0.151	
Gomphonema grovei var. *lingulatum* (Hustedt) Lange-Bertalot	0.398	2 (LM), 9 (SEM)
Gomphonema cf. *minutum* (Agardh) Agardh	0.246	2 (LM), 10 (SEM)
Gomphonema olivaceum (Hornemann) Kützing	0.192	
Gomphonema parvulum (Kützing) Kützing	0.217	2 (LM)
Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot	0.055	
Gomphonema truncatum Ehrenberg	0.071	
Gomphonemopsis obscura (Krasske) Lange-Bertalot	0.298	2 (LM), 10 (SEM)
Gyrosigma acuminatum (Kützing) Rabenhorst	0.014	
Gyrosigma nodiferum (Grunow) Reimer	0.015	
Gyrosigma sp.	0.067	
Halamphora aff. *tenerrima* (Aleem & Hustedt) Levkov	0.087	
Halamphora veneta (Kützing) Levkov	0.097	
Hantzschia virgata (Roper) Grunow	0.002	
Karayevia clevei (Grunow) Bukhtiyarova	0.022	
Luticola goeppertiana (Bleisch) D.G.Mann	0.087	

Diatom taxa	RA (%)	Plates
Luticola mutica (Kützing) D.G.Mann	0.031	
Luticula nivalis (Ehrenberg) D.G. Mann	0.001	
Mayamaea atomus (Kützing) Lange-Bertalot	0.022	
Melosira moniliformis (O.F.Müller) C.Agardh	0.031	
Melosira varians Agardh	0.347	2 (LM)
Navicula antonii Lange-Bertalot	1.215	3 (LM), 10 (SEM)
Navicula arenaria Donkin	0.027	
Navicula capitatoradiata Germain	0.213	3 (LM)
Navicula aff. *cari Ehrenberg*	0.004	
Navicula cryptotenella Lange-Bertalot	2.518	3 (LM), 11 (SEM)
Navicula cf. *cryptotenelloides* Lange-Bertalot	0.586	3 (LM), 11 (SEM)
Navicula erifuga Lange-Bertalot	0.016	
Navicula exigua Gregory	0.001	
Navicula exilis Kützing	0.002	
Navicula gregaria Donkin	0.540	3 (LM)
Navicula hintzii Lange-Bertalot	0.006	
Navicula aff. *molesta* Krasske	0.001	
Navicula aff. *mollis* (W. Smith) Cleve	0.720	3 (LM), 11 (SEM)
Navicula namibica Lange-Bertalot & Rumrich	0.023	
Navicula aff. *normaloides* Cholnoky	0.205	3 (LM)
Navicula aff. *pavillardi* Hustedt	0.061	
Navicula perminuta Grunow	1.128	3 (LM), 11 (SEM)
Navicula aff. *perminuta* Grunow	0.083	
Navicula cf. *perminuta* Grunow	1.250	3 (LM), 11 (SEM)
Navicula cf. *phylleptosoma* Lange-Bertalot	0.025	
Navicula radiosa Kützing	0.001	
Navicula recens (Lange-Bertalot) Lange-Bertalot	2.362	3 (LM), 12 (SEM)
Navicula aff. *recens* (Lange-Bertalot) Lange-Bertalot	0.268	3 (LM), 12 (SEM)
Navicula reichardtiana Lange-Bertalot	0.204	3 (LM)
Navicula salinarum Grunow	0.002	
Navicula salinicola Hustedt	0.049	
Navicula saprophila Lange-Bertalot & Bonik	0.066	
Navicula sp.	0.020	
Navicula tripunctata (O.F. Müller) Bory	0.357	3 (LM)
Navicula veneta Kützing	0.218	3 (LM)
Navicula viridula (Kützing) Kützing	0.077	
Nitzschia aff. *agnita* Hustedt	0.009	
Nitzschia amphibia Grunow	0.404	4 (LM)
Nitzschia amplectens Hustedt	0.017	
Nitzschia capitellata Hustedt	0.029	
Nitzschia cf. *clausii* Hantzsch	0.005	
Nitzschia coarctata Grunow	0.027	
Nitzschia constricta (Kützing) Ralfs	0.347	4 (LM), 12 (SEM)
Nitzschia dissipata (Kützing) Grunow	2.201	4 (LM), 12 (SEM)
Nitzschia filiformis (W. Smith) Van Heurck	0.899	4 (LM), 13 (SEM)
Nitzschia cf. *fonticola* (Grunow) Grunow	0.415	4 (LM)
Nitzschia frustulum [a] (Kützing) Grunow	8.187	4 (LM), 13 (SEM)

Diatom taxa	RA (%)	Plates
Nitzschia frustulum var. bulnheimiana (Rabenhorst) Grunow	0.811	4 (LM)
Nitzschia heufleriana Grunow	0.002	
Nitzschia inconspicua Grunow	5.438	4 (LM), 13 (SEM)
Nitzschia linearis (C.Agardh) W.Smith	0.002	
Nitzschia microcephala Grunow	0.185	4 (LM), 14 (SEM)
Nitzschia palea (Kützing) W. Smith	0.738	4 (LM), 14 (SEM)
Nitzschia cf. *palea* Grunow	0.601	4 (LM), 14 (SEM)
Nitzschia paleacea Grunow	0.176	
Nitzschia prolongata Hustedt	0.340	4 (LM)
Nitzschia recta Hantzsch ex Rabenhorst	0.048	
Nitzschia cf. *sociabilis* Hustedt	0.243	4 (LM)
Nitzschia cf. *solita* Hustedt	0.006	
Nitzschia sp.	0.001	
Nitzschia aff. *supralitorea Lange-Bertalot*	0.007	
Parlibellus cf. *berkeleyi (*Kützing) Cox	0.233	5 (LM)
Planothidium iberense Rovira & Witkowski	0.425	5 (LM), 14 (SEM)
Pleurosigma sp.	0.006	
Pleurosira laevis (Ehrenberg) Compère	0.238	5 (LM), 14 (SEM)
Psammothidium punctulatum (Simonsen) Bukhtiyarova et Round	0.297	5 (LM)
Psammothidium subatomoides (Hustedt) L.Bukhtiyarova & Round	0.004	
Pseudostaurosira brevistriata (Grunow) D.M.Williams & Round	0.031	
Pseudostaurosira elliptica (Schumann) Edlund, Morales & Spaulding	0.077	
Pseudostaurosira subsalina (Hustedt) E.A.Morales	0.073	
Reimeria uniseriata S.E.Sala, J.M.Guerrero & M.E.Ferrario	0.034	
Rhoicosphenia abbreviata (C. Agardh) Lange-Bertalot	7.241	5 (LM), 15 (SEM)
Seminavis cymbelloides (Grunow) D.G.Mann	0.383	
Seminavis sp.	0.123	
Stephanodiscus medius Håkansson	0.192	
Surirella brebisonii var. *brebisonii* Krammer & Lange-Bertalot	0.030	
Synedra ulna (Nitzsch) Ehrenberg	0.215	5 (LM), 15 (SEM)
Tabularia fasciculata (C. Agardh) Williams & Round	2.665	5 (LM), 15 (SEM)
Tabularia tabulata (C. Agardh) Snoeijs	1.589	5 (LM)
Thalassiosira bramaputrae (Ehrenberg) Håkansson & Locker	0.082	
Thalassiosira pseudonana Hasle & Heimdal	0.220	5 (LM)
Thalassiosira weissflogii (Grunow) G.Fryxell & Hasle	0.002	

[a]: The research in this thesis (Chapters 4 and 5) show that specimens identified in Chapters 1-3 as *N. frustulum* correspond to long cells of *N. inconspicua* after auxosporulation. However, they are named here as they are in the publications corresponding to Chapters 1-3.

LM and SEM plates of diatom taxa

Taxa are arranged alphabetically except *Eolimna subminuscula* (Manguin) Moser, Lange-Bertalot & Metzeltin, which is placed in Plate 3 (LM) and Plate 10 (SEM) due to its former name, i.e. *Navicula subminuscula* Manguin. Only diatom taxa with relative abundance (RA > 0.2%) and present in more than 5% of the Ebro Estuary samples are shown. LM pictures were obtained using differential interference contrast technique (DIC) with a 1000x magnification, unless otherwise stated. Scale bars are 10 μm for LM and 1 μm for SEM, except when specified.

Plate 1 LM

Figs.1-3. ***Achnanthes amoena*** Hustedt
Figs. 1, 2: raphe valve (RV)
Fig. 3: sternum valve (SV)

Figs. 4, 5. ***Achnanthes*** sp.
Fig. 4: RV
Fig. 5: SV

Figs. 6, 7. ***Achnanthidium minutissimum*** (Kützing) Czarnecki
Fig. 6: RV
Fig. 7: SV

Figs. 8, 9. ***Amphora inariensis*** Krammer
Fig. 8: valve
Fig. 9: frustule

Figs. 10-12. ***Amphora indistincta*** Levkov
Figs. 10, 11: valve
Fig. 12: frustule

Figs. 13, 14. ***Amphora* aff. *luciae*** Cholnoky
Fig. 13: frustule
Fig. 14: valve

Figs. 15-17. ***Amphora* cf. *meridionalis*** Levkov
Fig. 15: valve
Figs. 16, 17: frustule

Figs. 18, 19. ***Amphora ovalis*** (Kützing) Kützing
Fig. 18: valve
Fig. 19: frustule

Figs. 20, 21. ***Amphora pediculus*** (Kützing) Grunow, frustules

Figs. 22-24. ***Amphora polita*** Krasske, frustules

Figs. 25, 26. ***Amphora* cf. *vetula*** Levkov
Fig. 25: valve
Fig. 26: frustule

Fig. 27. ***Bacillaria paxillifera*** (O.F. Müller) Hendey, frustule

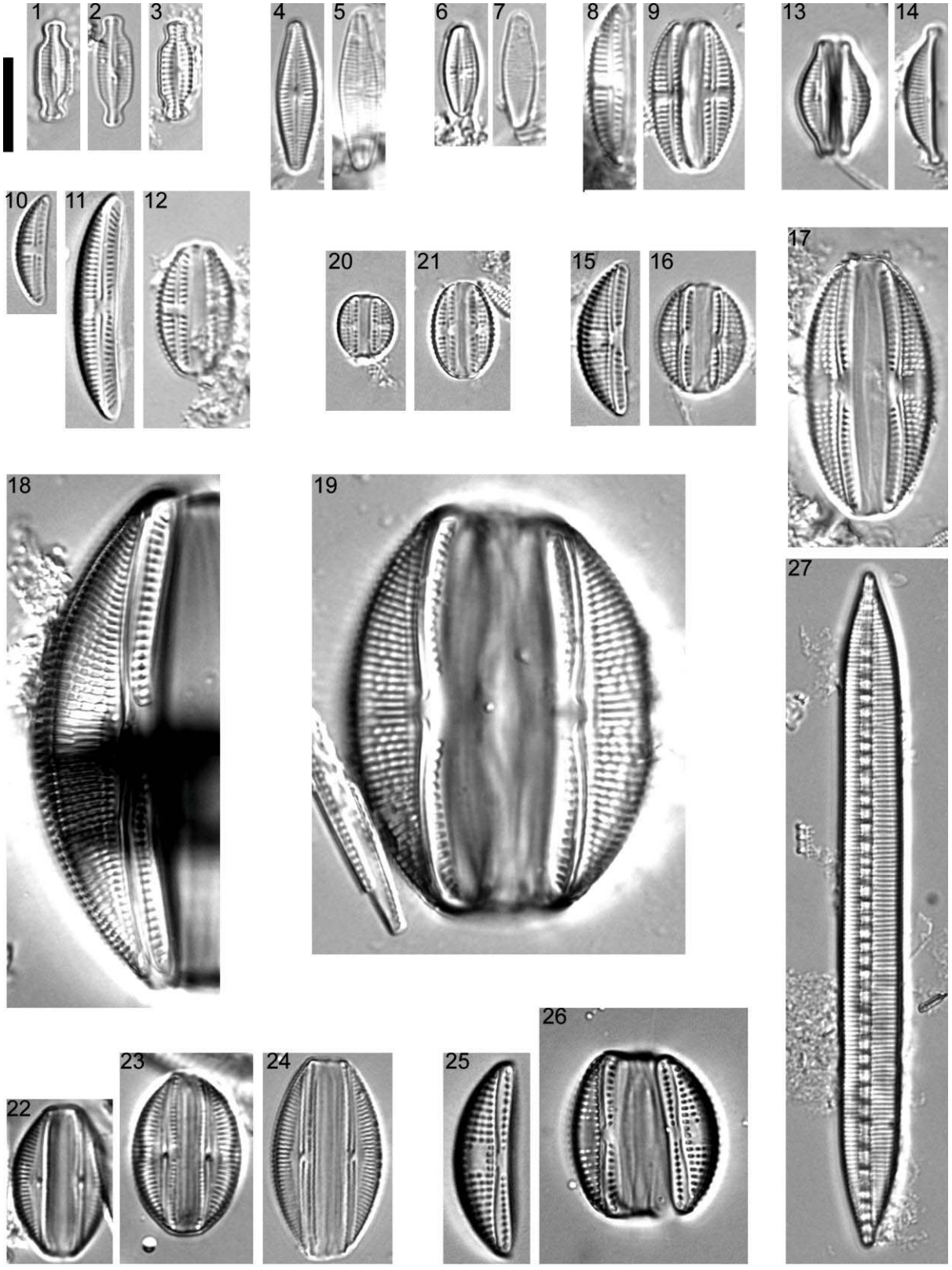
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Plate 2 LM

Figs. 1-3. ***Cocconeis* cf. *neothumensis* var. marina** De Stefano, Marino & Mazzella
Fig. 1: RV
Figs. 2, 3: raphe-sternum valve (RSV)

Figs. 4, 5. ***Cocconeis pediculus*** Ehrenberg, RSV

Fig. 6. ***Cocconeis placentula*** Ehrenberg, RV

Figs. 7, 8. ***Cocconeis placentula* var. *euglypta*** (Ehrenberg) Grunow, RSV

Fig. 9. ***Cocconeis placentula* var. *placentula*** Ehrenberg, RSV

Figs. 10-12. ***Cocconeis placentula* var. *trilineata*** (M. Peragallo & J. Héribaud) Cleve, RSV

Fig. 13. ***Cyclotella meneghiniana*** Kützing

Figs. 14-16 ***Diploneis* sp**.

Figs. 17-19. ***Fallacia clepsidroides*** Witkowski

Figs. 20, 21. ***Gomphonema grovei* var. *lingulatum*** (Hustedt) Lange-Bertalot

Figs. 22-24. ***Gomphonema* cf. *minutum*** (Agardh) Agardh

Figs. 25-27. ***Gomphonemopsis obscura*** (Krasske) Lange-Bertalot

Figs. 28, 29. ***Gomphonema parvulum*** (Kützing) Kützing

Fig. 30. ***Melosira varians*** Agardh

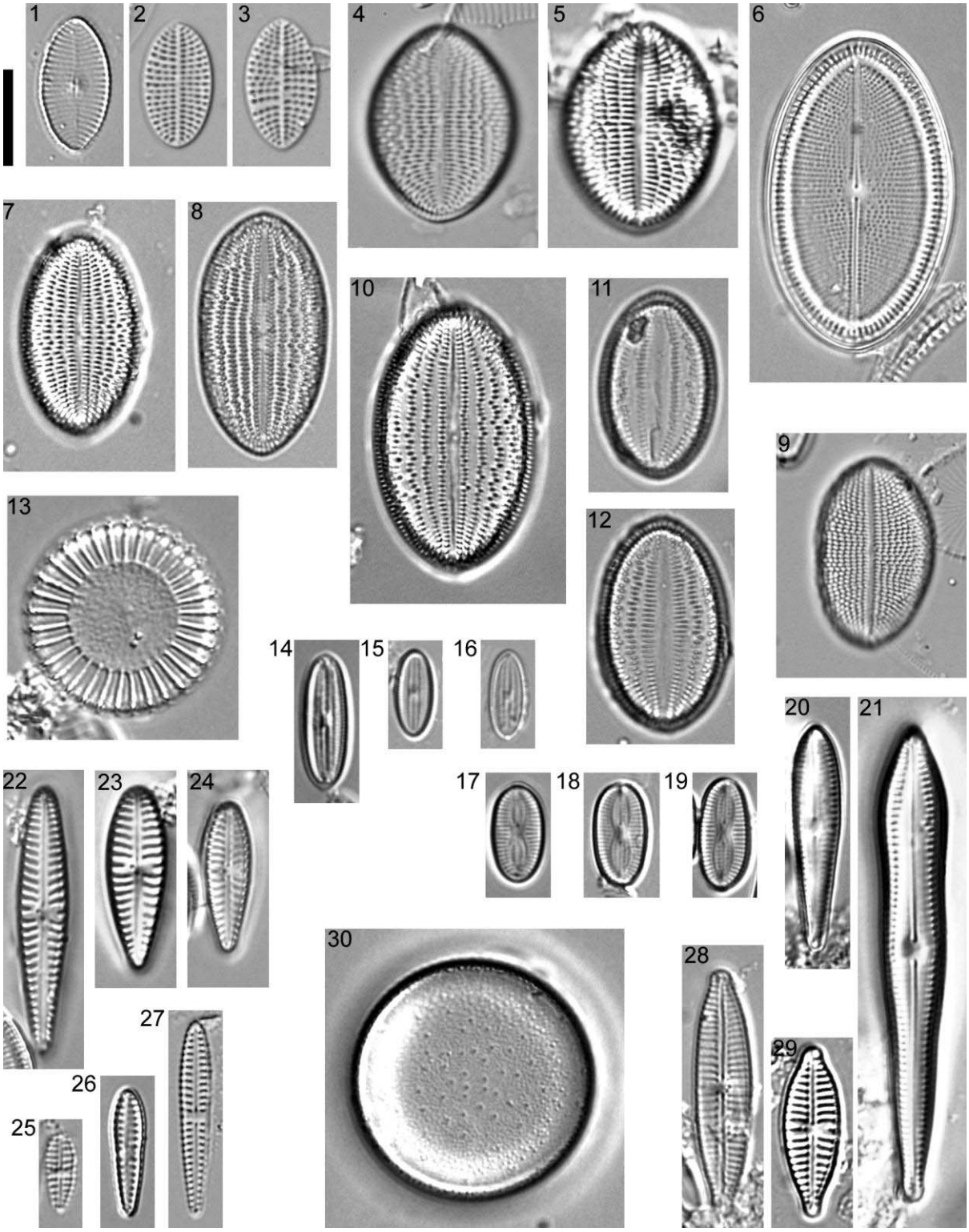
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Plate 3 LM

Figs. 1, 2. ***Navicula antonii*** Lange-Bertalot

Figs. 3, 4. ***Navicula capitatoradiata*** Germain

Figs. 5-8. ***Navicula cryptotenella*** Lange-Bertalot

Figs. 9-11. ***Navicula* cf. *cryptotenelloides*** Lange-Bertalot

Figs. 12, 13. ***Navicula gregaria*** Donkin

Figs. 14-16. ***Navicula* aff. *mollis*** (W. Smith) Cleve

Figs. 17, 18. ***Navicula* aff. *normaloides*** Cholnoky

Figs. 19-21. ***Navicula perminuta*** Grunow

Fig. 20: Bright field (BF)

Figs. 22-24. ***Navicula* cf. *perminuta*** Grunow

Figs. 25, 26. ***Navicula recens*** (Lange-Bertalot) Lange-Bertalot

Figs. 27-29. ***Navicula* aff. *recens*** (Lange-Bertalot) Lange-Bertalot

Figs. 30, 31. ***Navicula reichardtiana*** Lange-Bertalot

Fig. 32. ***Navicula tripunctata*** (O.F. Müller) Bory

Figs. 33, 34. ***Navicula veneta*** Kützing

Fig. 35. ***Eolimna subminuscula*** (Manguin) Moser, Lange-Bertalot & Metzeltin

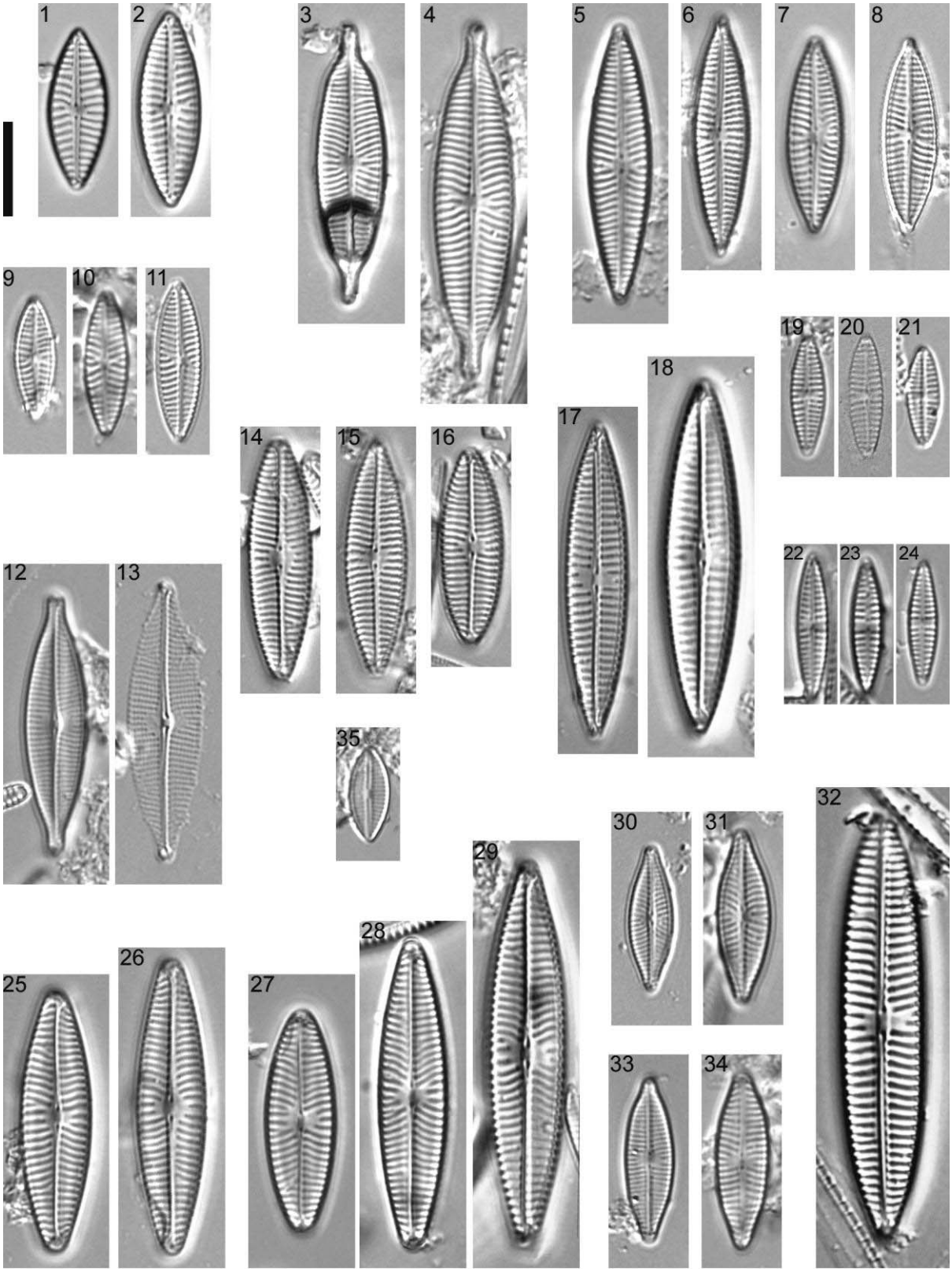
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Plate 4 LM

Fig. 1. ***Nitzschia amphibia*** Grunow

Fig. 2. ***Nitzschia constricta*** (Kützing) Ralfs

Figs. 3-5. ***Nitzschia dissipata*** (Kützing) Grunow

Figs. 6, 7. ***Nitzschia filiformis*** (W. Smith) Van Heurck

Figs. 8, 9. ***Nitzschia* cf. *fonticola*** (Grunow) Grunow

Figs. 10, 11. ***Nitzschia frustulum* var. *bulnheimiana*** (Rabenhorst) Grunow

Figs. 12- 14. ***Nitzschia inconspicua*** [a] Grunow, long cells

Figs. 15-17. ***Nitzschia inconspicua*** Grunow

Figs. 18-20. ***Nitzschia microcephala*** Grunow

Figs. 21-23. ***Nitzschia palea*** (Kützing) W. Smith

Figs. 24-27. ***Nitzschia* cf. *palea*** (Kützing) W. Smith

Fig. 28. ***Nitzschia prolongata*** Hustedt

Figs. 29-31. ***Nitzschia* cf. *sociabilis*** Hustedt

[a] Chapters 4 and 5 show that cells identified as *N. frustulum* in Chapters 1-3 (and in Appendix I) correspond to long cells of *N. inconspicua* after auxosporulation.

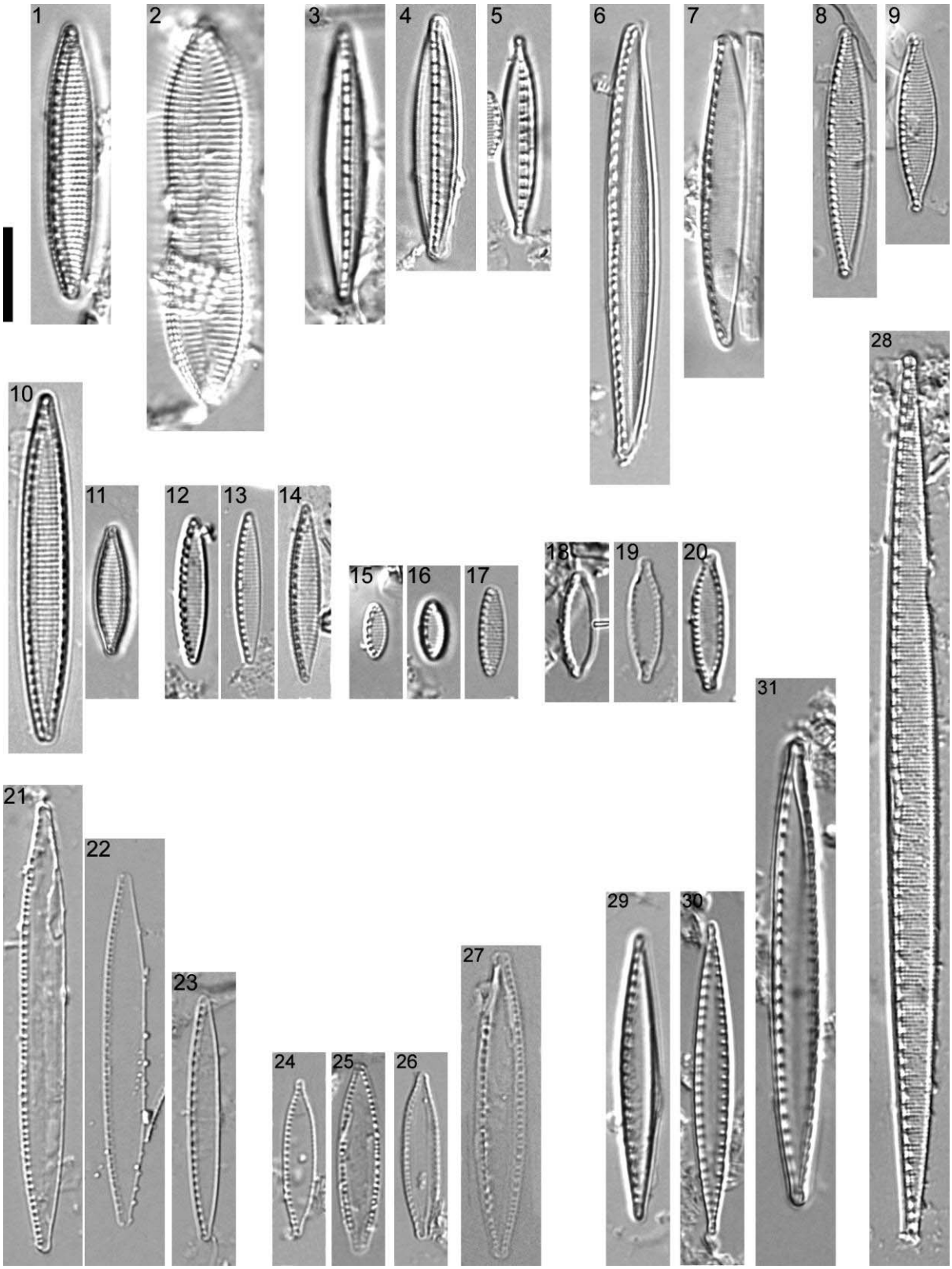
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Plate 5 LM

Figs. 1, 2. ***Parlibellus* cf. *berkeleyi*** (Kützing) Cox
Fig.1: frustule
Fig. 2: girdle view

Figs. 3-5. ***Planothidium iberense*** Rovira & Witkowski

Fig. 6. ***Pleurosira laevis*** (Ehrenberg) Compère

Figs. 7, 8. ***Psammothidium punctulatum*** (Simonsen) Bukhtiyarova et Round

Figs. 9-12. ***Rhoicosphenia abbreviata*** (Agardh) Lange-Bertalot
Fig. 9: girdle view
Fig. 10: SV
Figs. 11, 12: R

Figs. 13, 14. ***Synedra ulna*** (Nitzsch) Ehrenberg
Fig. 14: BF.

Fig. 15. ***Tabularia fasciculata*** (Agardh) Williams & Round

Figs. 16, 17. ***Tabularia tabulata*** (Agardh) Snoeijs

Fig. 18. ***Thalassiosira pseudonana*** Hasle & Heimdal

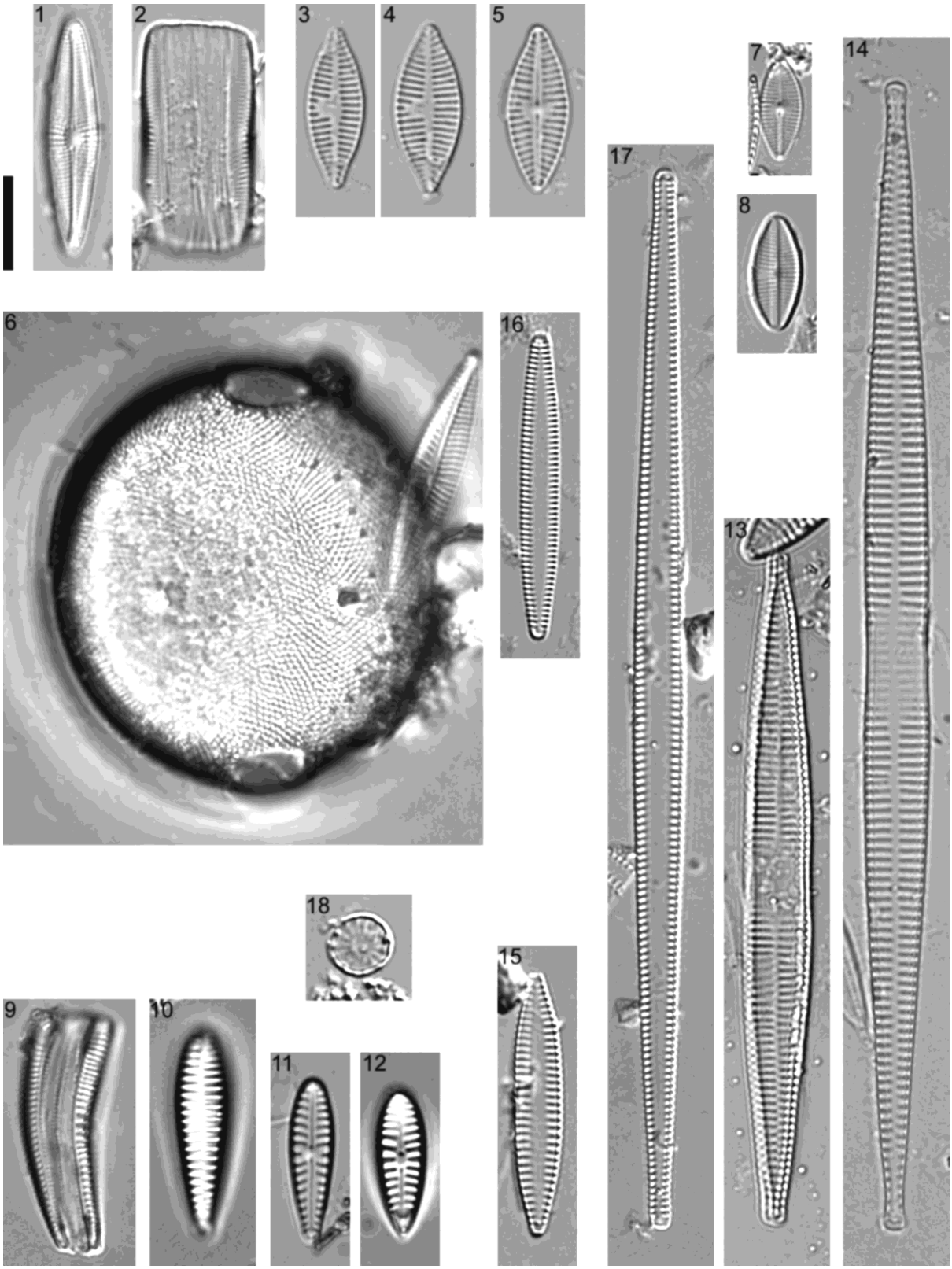
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Plate 6 SEM

Fig. 1. ***Achnanthidium minutissimum*** (Kützing) Czarnecki, RV

Fig. 2. ***Amphora inariensis*** Krammer, frustule ventral view

Fig. 3. ***Amphora indistincta*** Levkov, frustule ventral view

Fig. 4. ***Amphora* aff. *luciae*** Cholnoky, external ventral valve

Fig. 5. ***Amphora* cf. *meridionalis*** Levkov, frustule ventral view

Fig. 6. ***Amphora pediculus*** (Kützing) Grunow, frustule ventral view

Fig. 7. ***Amphora polita*** Krasske, external ventral valve view

Fig. 8. ***Amphora* cf. *vetula*** Levkov (scale bar: 5 µm), frustule ventral view

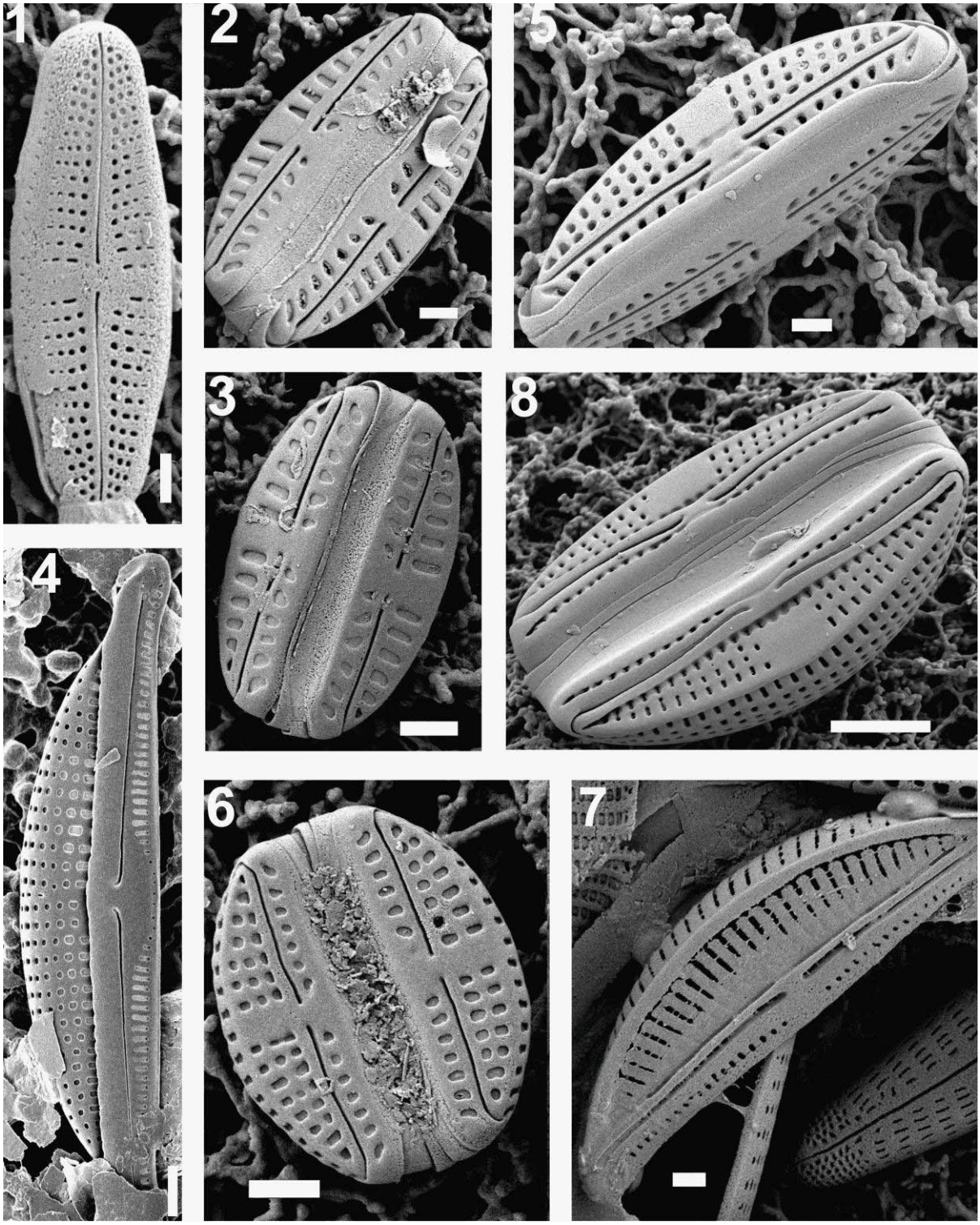
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Plate 7 SEM

Figs. 1-3. ***Cocconeis* cf. *neothumensis* var. marina** De Stefano, Marino & Mazzella

Fig. 1: internal RSV view

Fig. 2: internal RV view

Fig. 3: external RSV view

Figs. 4-6. ***Cocconeis pediculus*** Ehrenberg (scale bar: 5 µm).

Fig. 4: external RSV view

Fig. 5: internal RSV view

Fig. 6: internal RV view

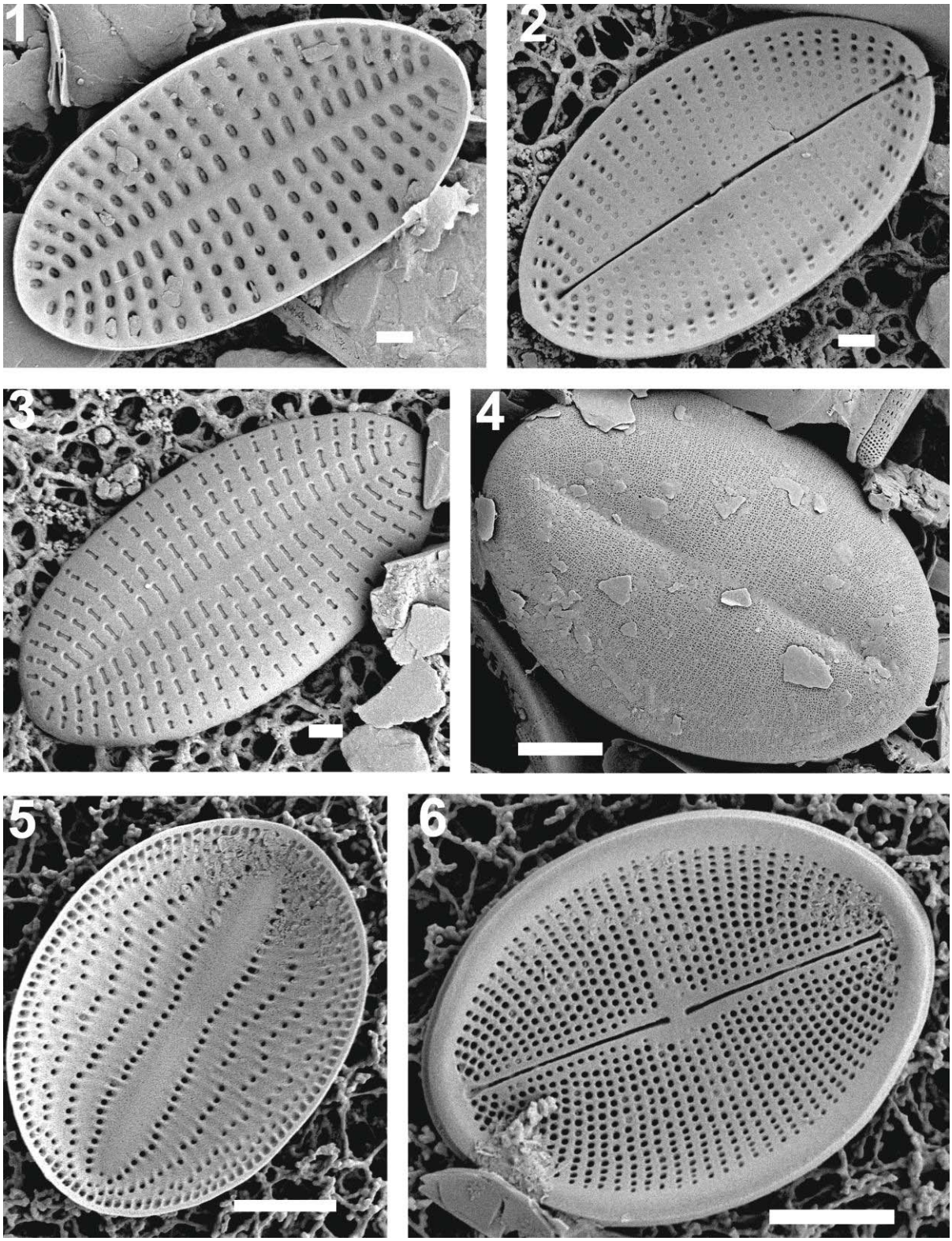
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Plate 8 SEM

Figs. 1, 2. ***Cocconeis placentula* var. *euglypta*** (Ehrenberg) Grunow

Fig. 1: external RSV view

Fig. 2: internal RV view

Figs. 3, 4. ***Cocconeis placentula* var. *placentula*** Ehrenberg

Fig. 3: internal RSV view

Fig. 4: internal RV view

Figs. 5, 6. ***Cocconeis placentula* var. *trilineata*** (M. Peragallo & J. Héribaud) Cleve

Fig. 5: internal RSV view

Fig. 6: external RSV view

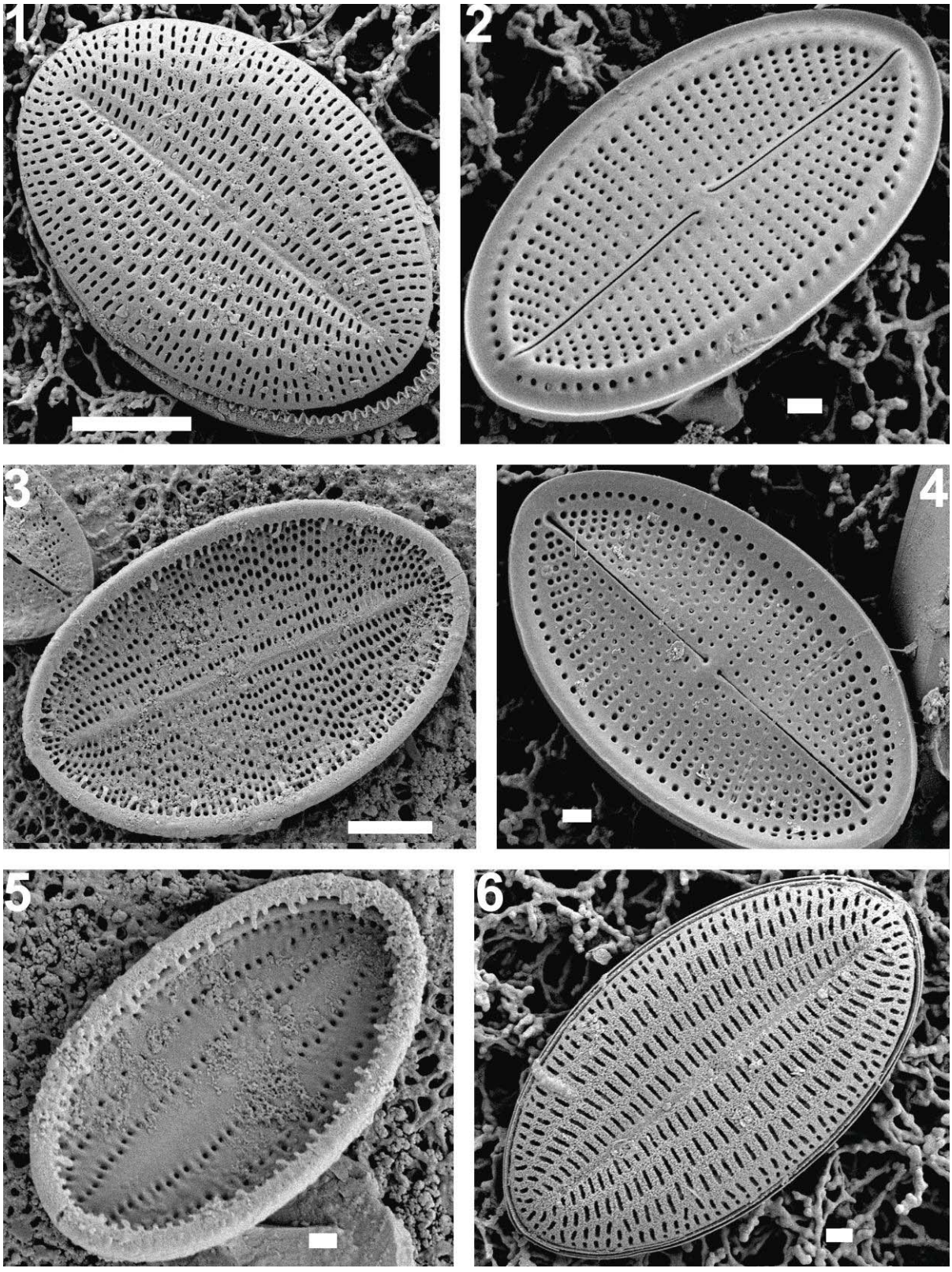
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Plate 9 SEM

Fig. 1. ***Cyclotella meneghiniana*** Kützing, external valve view

Fig. 2. ***Diploneis* sp.**, external valve view

Figs. 3, 4. ***Fallacia clepsidroides*** Witkowski

Fig. 3: external valve view

Fig. 4: internal valve view

Figs. 5, 6. ***Gomphonema grovei* var. *lingulatum*** (Hustedt) Lange-Bertalot

Fig. 5: external valve view

Fig. 6: internal valve view

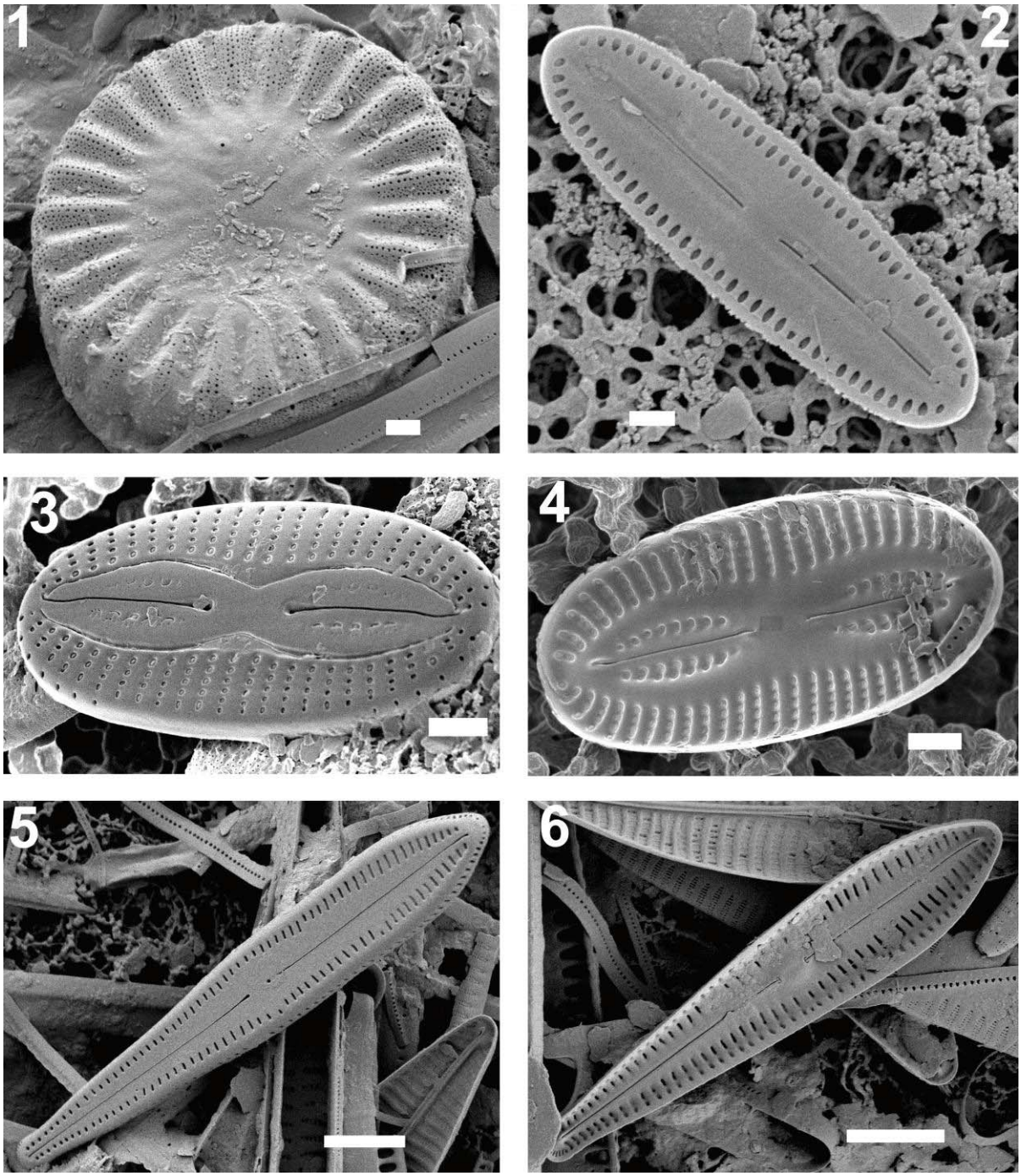
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Plate 10 SEM

Figs. 1, 2. ***Gomphonema* cf. *minutum*** (Agardh) Agardh (Scale bar: 5 µm)
Fig. 1: external valve view
Fig. 2: internal valve view

Figs. 3, 4. ***Gomphonemopsis obscura*** (Krasske) Lange-Bertalot
Fig. 3: external valve view
Fig. 4: internal valve view

Fig. 5. ***Eolimna subminuscula*** (Manguin) Moser, Lange-Bertalot & Metzeltin, internal valve view

Fig. 6. ***Navicula antonii*** Lange-Bertalot (Scale bar: 5 µm), frustule

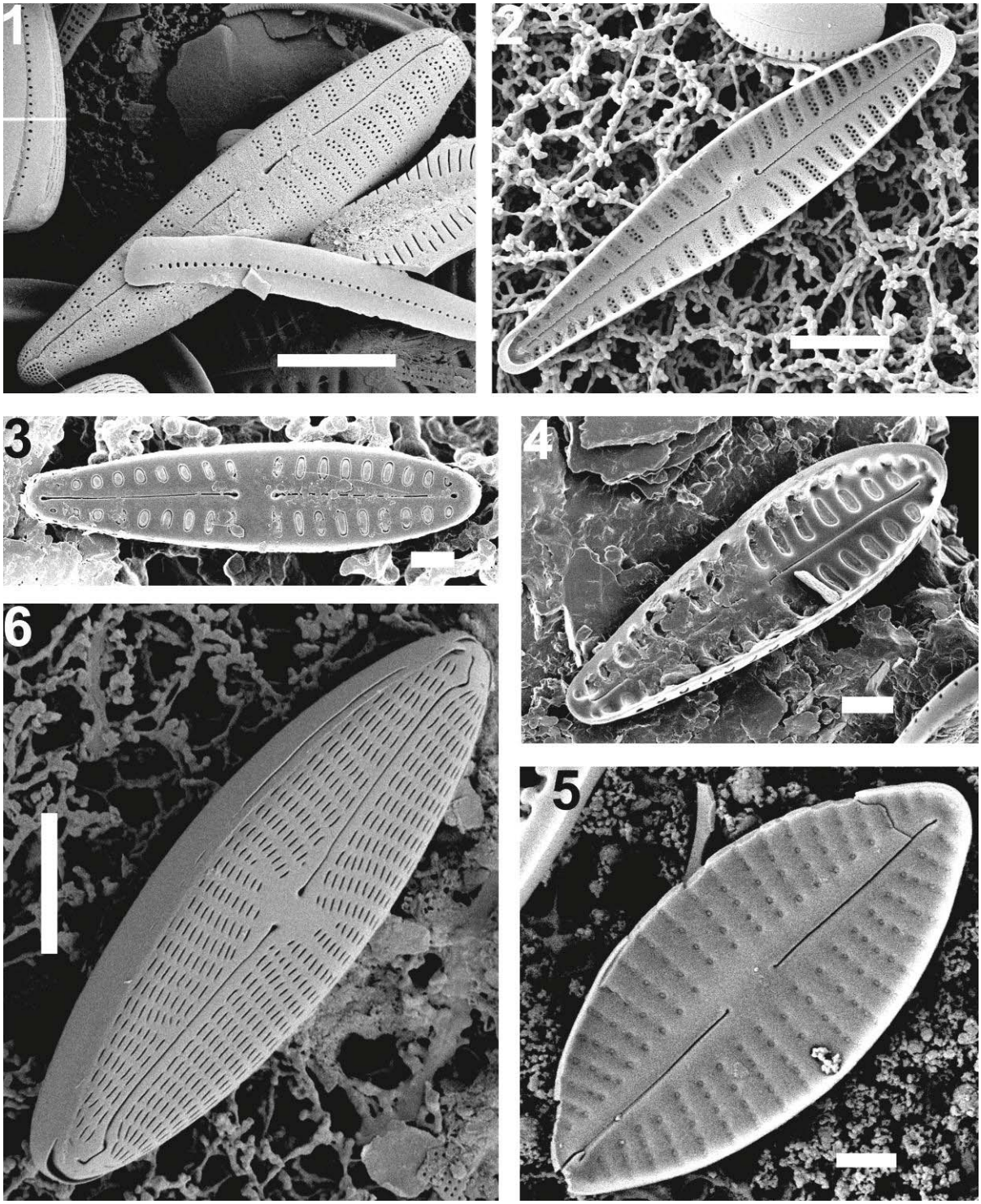
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Plate 11 SEM

Figs. 1, 2. ***Navicula cryptotenella*** Lange-Bertalot (scale bar: 5 µm)
Fig. 1: frustule
Fig. 2: internal valve view

Fig. 3. ***Navicula* cf. *cryptotenelloides*** Lange-Bertalot, frustule

Fig. 4. ***Navicula* aff. *mollis*** (W. Smith) Cleve, external valve view

Fig. 5. ***Navicula perminuta*** Grunow, external valve view

Fig. 6. ***Navicula* cf. *perminuta*** Grunow, external valve view

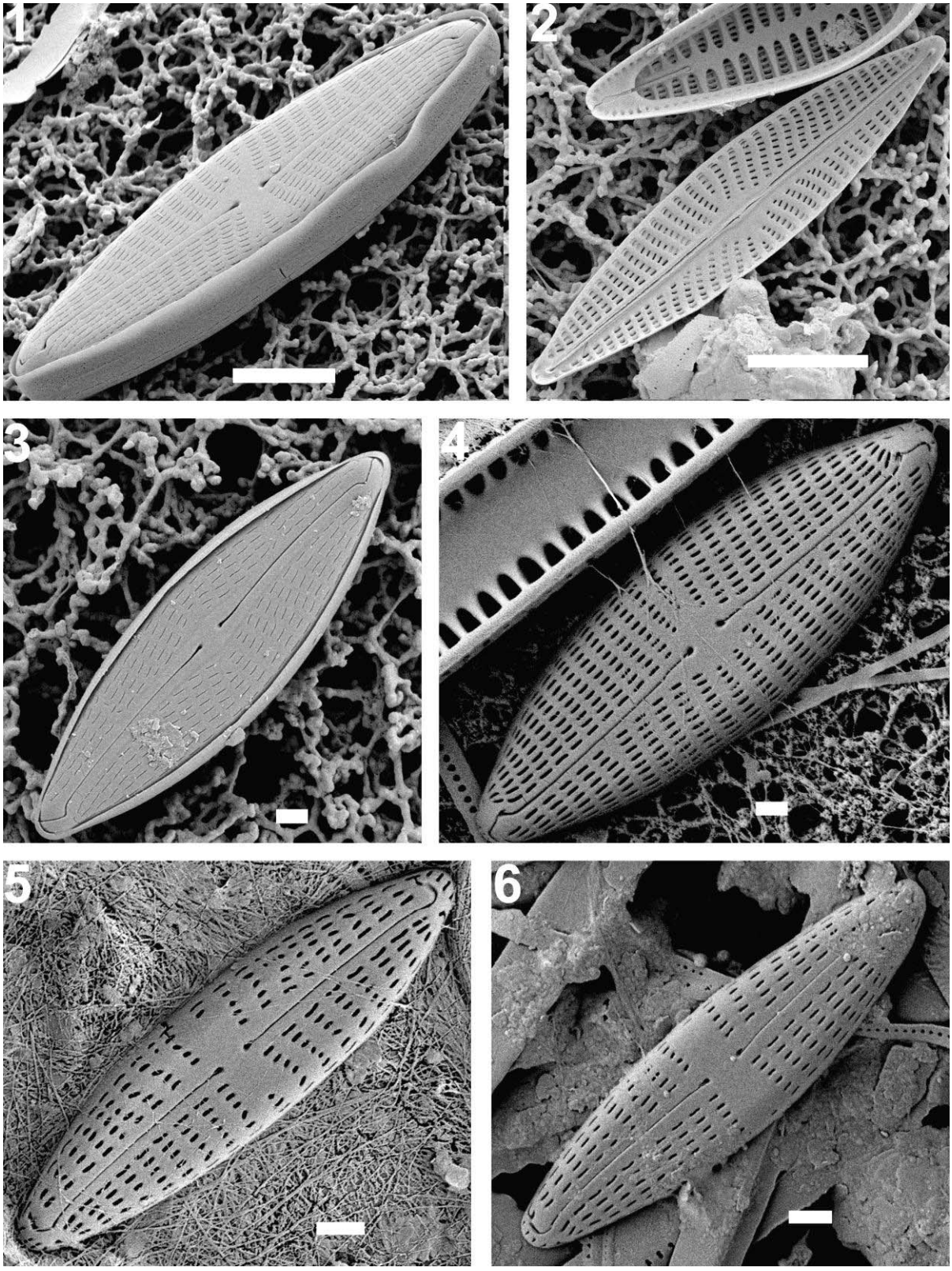
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Plate 12 SEM (Scale bar 5µm)

Figs. 1, 2. ***Navicula recens*** (Lange-Bertalot) Lange-Bertalot, external valve view

Figs. 3, 4. ***Navicula* aff. *recens*** (Lange-Bertalot) Lange-Bertalot, external valve view

Fig. 5. ***Nitzschia constricta*** (Kützing) Ralfs, internal valve view (Scale bar not available; check Fig. 2 in Plate 4 for LM valve)

Fig. 6. ***Nitzschia dissipata*** (Kützing) Grunow, internal valve view

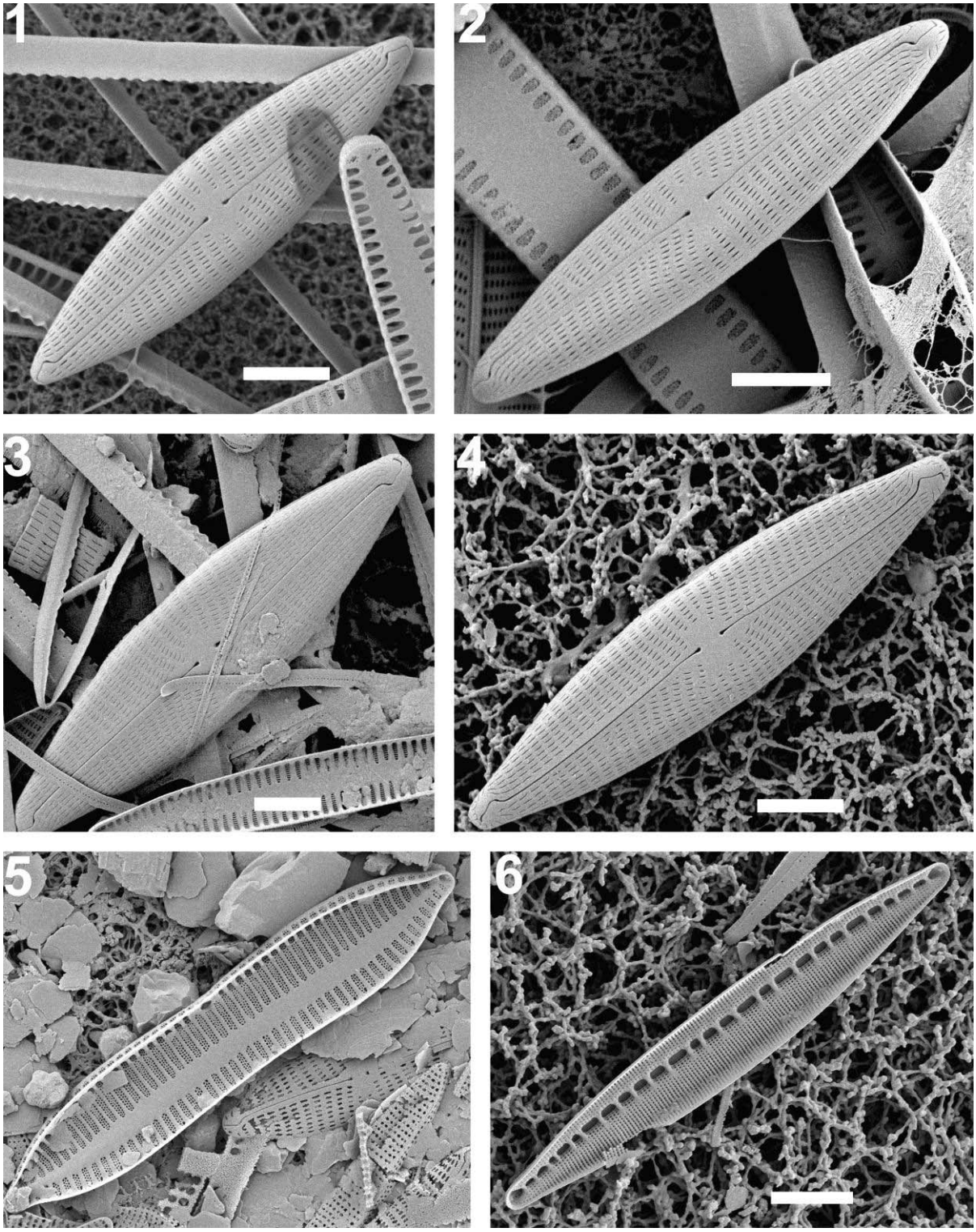
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Plate 13 SEM

Figs. 1, 2. ***Nitzschia filiformis*** (W. Smith) Van Heurck (scale bar: 5µm)

Fig. 1: external valve view

Fig. 2: detail of central raphe endings

Figs. 3-7. ***Nitzschia inconspicua*** Grunow

Fig. 3: external large valve view

Fig. 4: detail of straight central raphe endings

Fig. 5: external small valve view, deflected central raphe endings

Fig. 6: internal small valve view

Fig. 7: Detail of straight central raphe endings

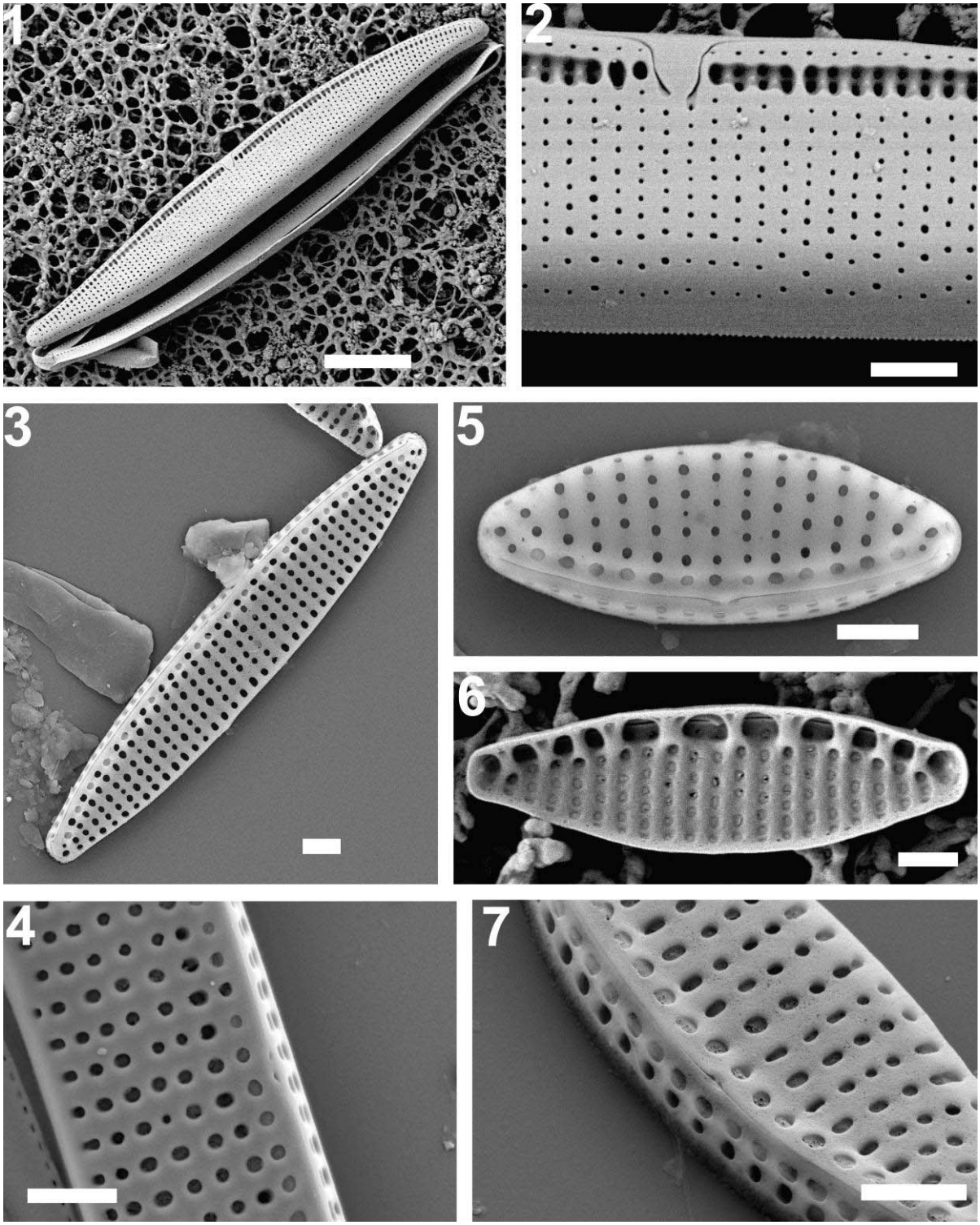
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Plate 14 SEM

Fig. 1. ***Nitzschia microcephala*** Grunow, internal valve view

Fig. 2. ***Nitzschia palea*** (Kützing) W. Smith (scale bar: 5µm), frustule

Fig. 3. ***Nitzschia* cf. *palea*** (Kützing) W. Smith, frustule

Fig. 4, 5. ***Planothidium iberense*** Rovira & Witkowski

Fig. 4: External RV view

Fig. 5: External SV view

Fig. 6. ***Pleurosira laevis*** (Ehrenberg) Compère (scale bar: 5µm), external view

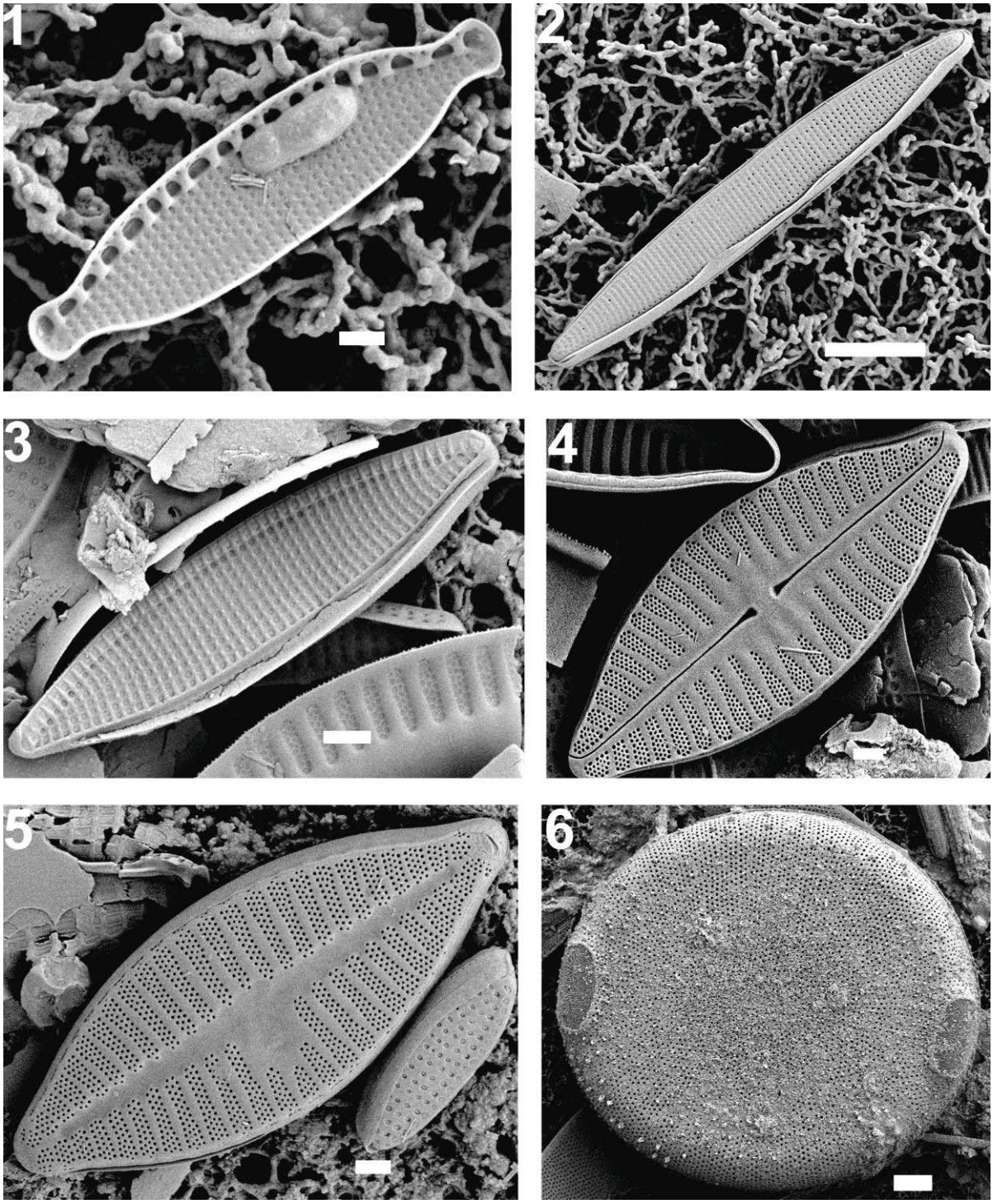
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Plate 15 SEM

Figs. 1-3. ***Rhoicosphenia abbreviata*** (Agardh) Lange-Bertalot

Fig. 1: External RV view

Fig. 2: External SV view

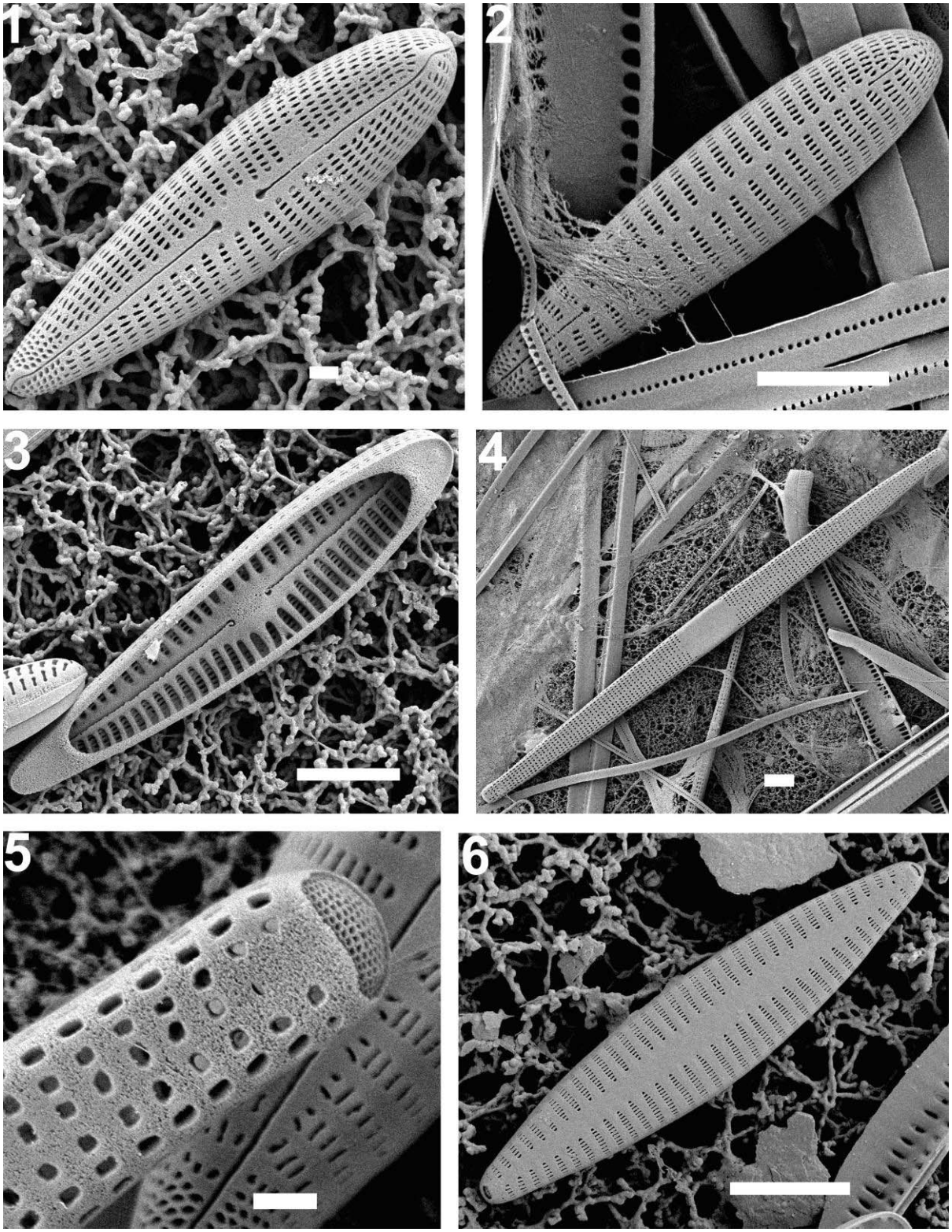
Fig. 3: Internal RV view

Figs. 4, 5**.** ***Synedra ulna*** (Nitzsch) Ehrenberg, external valve view

Fig. 6. ***Tabularia fasciculata*** (Agardh) Williams & Round (scale bar: 5µm)

Fig. 5: Detail of the ocellulimbus

Fig. 6: External valve view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Appendix

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Periphytic diatom community in a Mediterranean salt wedge estuary: the Ebro Estuary (NE Iberian Peninsula)

LAIA ROVIRA*, ROSA TROBAJO, CARLES IBÁÑEZ

IRTA – Aquatic Ecosystems, Carretera Poble Nou KM 5.5, 43540, St. Carles de la Ràpita, Catalonia, Spain.

The Ebro River discharge is the main factor controlling hydrological dynamics in the Ebro microtidal salt wedge estuary. The aim of this study was to describe the species composition of the periphytic diatom communities, and to elucidate the main environmental factors affecting them. Samples of periphytic diatoms were collected at 8 sites along the estuary in October 2007 and January 2008. The diatom community was sampled both from natural and artificial substrata. Water depth, velocity, pH, dissolved oxygen, temperature, conductivity, total chlorophyll and water chlorophyll *a*, total periphytic chlorophyll, dissolved nutrients ($P\text{-}PO_4^{3-}$, $N\text{-}NO_3^-$, $N\text{-}NO_2^-$, $N\text{-}NH_4^+$, $Si\text{-}SiO_4^{4-}$), total dissolved nitrogen and total dissolved phosphorous were determined in each campaign. Altogether, more than 120 taxa of diatoms were identified. The most abundant genera were *Cocconeis*, *Amphora*, *Navicula* and *Tabularia*. The variability of the diatom community was analyzed with multivariate analysis methods. Water stratification affected diatom community in both the horizontal and the vertical gradient, according mainly to salinity, dissolved oxygen and nutrient concentration differences.

Key words: Diatom, estuary, salt wedge, periphyton, distribution, taxonomic composition, Ebro, Mediterranean, Spain

Introduction

Diatoms are valuable indicators of ecological quality: they respond directly and sensitively to many physical, chemical and biological changes in aquatic environment. They are found in almost all aquatic habitats and their high contribution to primary production has been pointed out by several authors. They also have among the shortest generation times of all biological indicators, allowing them to respond rapidly to environmental changes and to provide early warning of potential changes in nutrient status for different water bodies (ROTT 1991, STEVENSON and PAN 1999).

* Corresponding author: laia.rovira@irta.cat

Despite the ecological importance of diatoms as a periphytic component, the knowledge of lentic communities is less than the knowledge of lotic periphytic communities. The scarcity of diatom studies in estuarine and transitional water bodies may be caused by the complexity of these systems (with high fluctuations among environmental parameters), but also due to the taxonomical difficulty of identifying estuarine diatom flora (TROBAJO et al. 2004). However, this fluctuating dynamics is interesting to study because it may help us to understand the responses of diatoms to environmental gradients. Nowadays these studies are focused mainly in tidal estuaries and there is a lack of knowledge of periphytic communities in stratified estuaries (with small tidal effect).

The Ebro Estuary is classified as a Mediterranean salt wedge or highly stratified estuary (IBÁÑEZ et al. 1997), with two completely different water layers and low tidal range (around 20 cm). The Ebro River discharge is the main factor that affects the dynamics of the salt wedge, but other factors like the topography of the estuarine bed must be also considered. The river discharge has been highly regulated by dams built since the 1960s, which decreased the annual Ebro River discharge and, therefore, increased the presence of the salt wedge. This stratification in two water layers has an ecological significance since biological communities in the estuary will receive either freshwater or saline water, depending on the dynamics of the salt wedge. The Ebro River has also great socioeconomic importance since it is the largest river in Spain and its flow is the water source for irrigation in the Ebro basin (IBÁÑEZ et al. 1996).

Although during the last 20 years a number or research projects concerned with the Ebro River and its estuary have produced a large amount of ecological and hydrological data of these systems (MUÑOZ and PRAT 1989, GUILLÉN and PALANQUES 1992, IBÁÑEZ et al. 1999, SIERRA et al. 2002, 2004, FALCÓ et al. 2006), there have been no studies on its periphytic diatom communities.

The aim of this preliminary study was to carry out the first description of the periphytic diatom community in the Ebro Estuary and to explore the main factors that may affect their distribution. This is an initial analysis as a part of a broader study of diatom community of Mediterranean estuarine systems.

Materials and methods

Study site

The Ebro River is the largest river in Spain. Its estuary covers an approximate area of 7 km^2 and it is considered a »micro-tidal salt wedge estuary«. Tides have little or no influence because of the low tidal range (around 20 cm), the Ebro River discharge being the main factor controlling the hydrological dynamics of this transitional system.

The Ebro Estuary is 30 km long with a mean depth of 6.8 m and a mean width of 237 m. The microtidal range (around 20 cm), favours the stratification of the water column and the existence of a salt wedge, with a maximum intrusion into the Ebro river channel of 32 km. This salt wedge disappears when the river flow is above 400 m^3s^{-1}. When the river discharge is between 400 and 300 m^3s^{-1}, the salt wedge can occupy the last 5 km of the estuary, but with discharges lower than 300 m^3s^{-1} the salt wedge advances quickly up to 18 km from the mouth. When the river discharge is less than 100 m^3s^{-1} , the salt wedge reaches its maximum extent (i.e. 32 km from the mouth) (IBÁÑEZ et al. 1997).

Sampling

Eight sampling points were established along the estuary (Fig. 1). The distance between sampling points was approximately 5 km. The first point was above the maximum extent of the salt wedge (E-3), thus it did not receive saline water, and the last point was located at the river mouth.

Every site was sampled in October 2007 and January 2008. Water depth, temperature, electrical conductivity (EC_{25}), dissolved oxygen (DO_2) and pH were measured in situ in all sampling sites with an YSI 556 multiprobe. Flow direction and velocity were also measured using a Braystoke BFM 001 current flow meter. Irradiance was measured with a QSP-2100 Submersible Scalar PAR Sensor.

Periphytic samples were collected from two different substrata: natural substratum (mainly macrophytes *Potamogeton pectinatus* and *Ceratophyllum* sp., but also wood debris where macrophytes were not available) and from artificial substrata (fired clay bricks, Fig. 2), to avoid variability due to substratum. A known area of periphyton (4 cm^2) was scraped with a brush from the artificial substrata in each replicate. Three fragments from natural substrata were included in each replicate. In order to study the effects of the salt wedge on diatom communities, bricks were placed in the superficial water layer (0.5 m water depth) and in the deep-water layer (which ranged from 2–8 m). Bricks were considered the most appropriate artificial substratum due to their resistance to high flows and to the sudden variations of flows that characterize the lower Ebro River. Unfortunately, due to this flow dynamics, in some sites, artificial substrata were not encountered. Two replicates from superficial and deep water bricks (when they were available) were processed. Two replicates from natural superficial substrata were also processed.

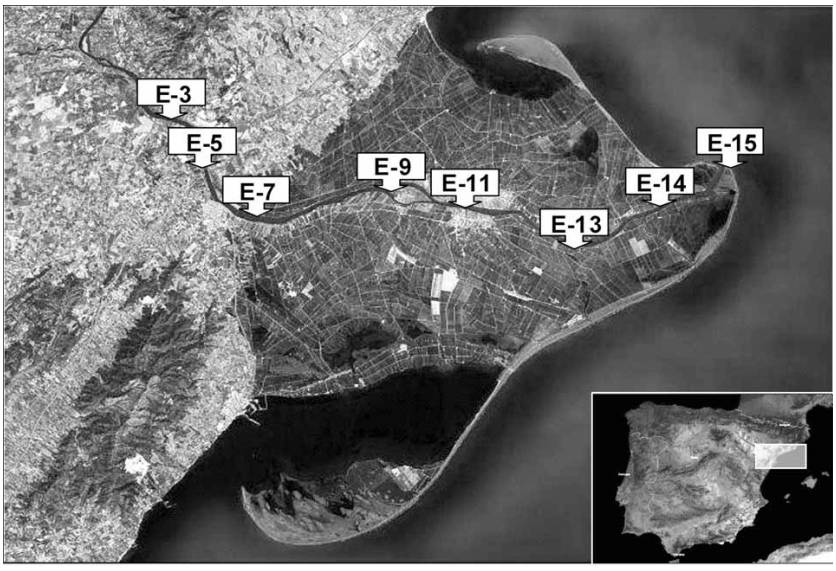

Fig. 1. Ebro estuary map showing the sampling points.

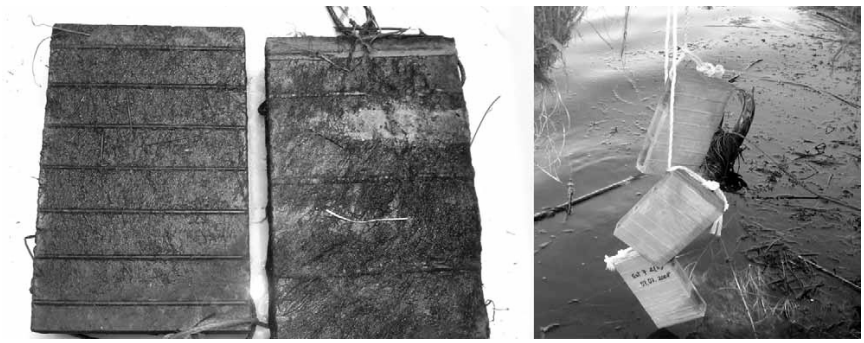

Fig. 2. Artificial substrata (fired clay bricks) used for diatom colonization both in superficial and deep-water layers.

Diatom identification and valve counting

The periphytic samples were cleaned of organic material using distilled water, H_2SO_4 and KNO_3 (HUSTEDT 1930). Clean valves were permanently mounted in Naphrax® (refractive index 1.74). The permanent slides were examined using a LEICA DMI 3000B light microscope equipped with differential interference contrast (DIC) with a 100 times oil immersion objective (n.a.=1.40). For the scanning electron microscopy (SEM) observations, the cleaned material was gold coated and studied under a JEOL – 6400 SEM.

The relative abundance of species was determined by counting a minimum of 400 frustules in each substratum replicate. Identification of diatoms to species level was based mainly on appropriate keys (KRAMMER and LANGE-BERTALOT 1991a, b; 1997a, b; WITKOWSKI et al. 2000; LANGE-BERTALOT 2001).

Nutrient and chlorophyll analysis

Analysis of dissolved inorganic nutrients: silicate ($Si\text{-}SiO_4^{4-}$), nitrate ($N\text{-}NO_3^-$), nitrite ($N\text{-}NO_2^-$), phosphate ($P\text{-}PO_4^{3-}$); total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were measured following GRASSHOFF et al. (1999), while ammonium ($N\text{-}NH_4^+$) was measured following the method proposed by the equipment manufacturer, ALLIANCE INSTRUMENTS, SA.

Total periphytic chlorophyll and water chlorophyll were extracted using 90% acetone and measured with a spectrophotometer using Jeffrey and Humphrey expressions (ROWAN 1989).

Data analysis

Diatom community variation along the Ebro estuary was analysed with a correspondence analysis (CA) using CANOCO 4.5 version with diatom relative abundance. To avoid the effect of rare species, only diatom species with a relative abundance (RA > 0.2%) and present in more than 10% of the samples (total number of samples = 32) were included in the analysis. Relative abundance data was square-root transformed in order to reduce the effect of highly variable population densities on ordination scores. Relationships between

CA dimensions and environmental parameters were determined with Pearson correlations using the SPSS 15.0 software for Windows. Only correlations with $P < 0.05$ were considered. Environmental data (except pH, water depth and water velocity) were logarithmically transformed before analysis.

Results

Physical and chemical parameters

The average values for the water physicochemical parameters measured in October 2007 and January 2008 at each sampling point are shown (Tab. 1).

Mean monthly river discharge was 175.95 $m^3 s^{-1}$ in October and 110.41 $m^3 s^{-1}$ in January.

In both sampling periods the salt wedge was detected up to the E-9 site (Fig. 1). In October we found and sampled the limit of the salt wedge, whereas in January the salt wedge was detected further upstream (between E-7 and E-9 deep water layers). The superficial layer was in both sampling periods oligohaline; salinity ranged approximately from 0.70 to near 3.00, whereas in the deep-water layer salinity ranged from oligohaline (0.70–0.80) in E-3, E-5 and E-7 to euhaline (36.00–37.00) in E-9, E-11, E-13, E-14 and E-15.

Irradiance values were 37 $\mu E\ m^{-2}\ s^{-1}$ at 6 m depth and 1104 $\mu E\ m^{-2}\ s^{-1}$ at surface (October); in January values ranged from 44 $\mu E\ m^{-2}\ s^{-1}$ at 7 m depth to 1407 $\mu E\ m^{-2}\ s^{-1}$ at surface.

Dissolved oxygen concentration (DO_2) was lower in October. In both sampling periods, DO_2 concentration was lower in the salt wedge than in the superficial freshwater layer and E-9D showed the lowest DO_2 concentrations among all sampling points.

Nutrients were usually higher in the freshwater layer than in the salt wedge. It should be noted that for both sampling periods, E-9D presented the highest nutrient concentrations among all the salt wedge sites (except in the case of $N\text{-}NO_3^-$ in October 2007) and the lowest values for pH and DO_2 among all sampling points (considering both freshwater layer and salt wedge). E-9D also showed the highest values of conductivity, $P\text{-}PO_4^{3-}$, TDP and $N\text{-}NH_4^+$ among all sampling points in October.

Chlorophyll

The highest values of both water chlorophyll *a* and total water chlorophyll concentrations (Tab. 2) were found in the sampling points where the salt wedge was present (E-9, E-11, E-13, E-14 and E-15). The average minimum water chlorophyll *a* values were 0.87 $\mu g\ L^{-1}$ in the freshwater layer and 0.79 $\mu g\ L^{-1}$ in the salt wedge, both in January. Minimum water total chlorophyll values were 1.10 $\mu g\ L^{-1}$ in the freshwater layer and 1.24 $\mu g\ L^{-1}$ in the salt wedge, both in January. Periphytic total chlorophyll ($a + b + c$) was always higher in superficial samples than in the deep ones, independently of the presence of the salt wedge (Tab. 2).

Diatom community

Altogether, 122 diatom species were identified in the 32 analysed samples. The most abundant genera (considering all species) were *Cocconeis* (24%), *Navicula* (21%), *Nitzschia* (17%) and *Tabularia* (11%). *Navicula* was the genus with the highest number of taxa

Tab. 1. Water physicochemical parameters measured in October 2007 and January 2008. The negatives values in water velocity mean that water flowed in the opposite direction to river flow. S – superficial water layer, D – deep water layer

Sampling point	Water depth (m)	Temp (°C)	pH	DO_2 (mg/L)	Salinity (psu)	Conductivity (µS/cm)	P-PO_4^{3-} (mg/L)	TDP (mg/L)	N-NH4 (mg/L)	N-NO2 (mg/L)	N-NO_3^- (mg/L)	TDN (mg/L)	Si-SiO_4^{4-} (mg/L)	Water velocity (m/s)
October 2007														
E-3S	0.2	22.8	8.2	7.1	0.72	1375.0	0.028	0.067	0.086	0.014	1.79	2.46	0.85	0.34
E-3D	1.0	22.8	8.2	6.9	0.72	1374.4	0.029	0.070	0.028	0.014	1.63	2.40	0.65	0.16
E-5S	0.2	22.9	8.3	7.9	0.72	1373.7	0.041	0.077	0.070	0.014	1.75	2.35	0.76	0.17
E-5D	7.0	22.8	8.2	7.0	0.72	1367.9	0.028	0.066	0.075	0.013	1.73	2.50	0.79	0.12
E-7S	0.2	22.9	8.3	8.1	0.72	1373.8	0.021	0.052	0.127	0.011	1.68	2.38	1.31	0.19
E-7D	3.0	22.9	8.3	8.1	0.72	1372.6	0.024	0.058	0.061	0.011	1.70	2.42	1.04	0.17
E-9S	0.2	23.0	8.4	8.9	0.73	1408.0	0.022	0.050	0.075	0.012	1.75	2.34	1.24	0.28
E-9D	4.0	23.7	8.0	2.2	35.41	52248.4	0.049	0.095	0.295	0.014	0.04	0.40	0.91	–0.03
E-11S	0.2	23.2	8.4	8.9	1.09	2053.9	0.022	0.049	0.081	0.012	1.65	2.34	1.87	0.31
E-11D	6.0	21.5	8.3	5.5	35.80	50474.1	0.013	0.057	0.080	0.002	0.05	0.19	0.22	–0.07
E-13S	0.2	22.8	8.3	8.6	1.59	2917.8	0.017	0.044	0.031	0.013	1.77	2.26	2.61	0.39
E-13D	7.0	22.3	8.3	6.4	36.25	51887.9	0.006	0.042	0.017	0.000	0.03	0.16	0.08	–0.05
E-14S	0.2	22.5	8.4	9.1	1.98	3577.3	0.013	0.043	0.073	0.012	1.68	2.56	2.52	0.45
E-14D	6.8	22.1	8.4	7.8	36.42	51823.3	0.003	0.039	0.024	0.000	0.02	0.14	0.63	–0.14
E-15S	0.2	22.3	8.3	8.5	2.89	5074.8	0.016	0.047	0.047	0.012	1.56	2.08	2.08	0.47
E-15D	4.5	21.9	8.4	8.2	36.00	51139.7	0.003	0.037	0.022	0.001	0.06	0.12	0.52	–0.23

Tab. 1. continued

Sampling point	Water depth (m)	Temp (°C)	pH	DO_2 (mg/L)	Salinity (psu)	Conductivity (µS/cm)	$P\text{-}PO_4^{3-}$ (mg/L)	TDP (mg/L)	N-NH4 (mg/L)	N-NO2 (mg/L)	$N\text{-}NO_3^-$ (mg/L)	TDN (mg/L)	$Si\text{-}SiO_4^{4-}$ (mg/L)	Water velocity (m/s)
January 2008														
E-3S	0.2	11.0	7.8	13.7	0.76	1105.9	0.050	0.064	0.024	0.019	3.49	3.77	0.99	0.27
E-3D	3.0	11.0	7.9	13.2	0.76	1105.6	0.038	0.061	0.024	0.018	3.51	3.75	1.06	0.18
E-5S	0.2	11.7	7.9	13.7	0.74	1093.9	0.029	0.055	0.104	0.018	3.54	4.07	0.87	0.12
E-5D	7.0	11.4	7.9	13.7	0.75	1098.5	0.030	0.046	0.045	0.017	3.50	3.87	0.80	0.10
E-7S	0.2	11.6	8.0	16.5	0.78	1142.9	0.021	0.036	0.022	0.017	3.34	3.70	0.92	0.15
E-7D	3.0	11.4	8.0	14.8	0.79	1156.4	0.015	0.038	0.019	0.017	3.46	3.74	1.10	0.11
E-9S	0.2	11.7	8.0	17.7	0.91	1335.4	0.027	0.045	0.007	0.021	3.53	3.93	1.34	0.27
E-9D	5.0	13.3	7.9	9.0	36.36	42631.2	0.029	0.054	0.089	0.015	0.13	0.27	0.45	0.00
E-11S	0.2	11.9	8.1	19.1	1.34	1932.9	0.020	0.045	0.027	0.022	3.69	3.86	1.05	0.29
E-11D	6.0	13.3	8.0	11.3	36.60	42892.7	0.009	0.020	0.042	0.008	0.09	0.14	0.16	0.00
E-13S	0.2	11.8	8.0	15.6	1.63	2322.8	0.026	0.050	0.031	0.023	3.45	3.81	0.77	0.35
E-13D	7.0	13.2	8.0	10.4	36.78	43053.6	0.004	0.015	0.038	0.006	0.09	0.13	0.10	–0.03
E-14S	0.2	11.8	8.1	20.5	1.84	2604.0	0.031	0.048	0.040	0.023	3.42	3.78	0.92	0.65
E-14D	8.0	13.3	8.0	10.6	37.34	43688.1	0.002	0.015	0.036	0.004	0.11	0.16	0.08	–0.06
E-15S	0.2	11.5	8.0	13.6	2.57	3547.3	0.037	0.051	0.049	0.023	3.24	3.70	0.66	0.55
E-15D	4.0	13.3	8.0	10.4	37.36	43776.1	0.001	0.012	0.029	0.003	0.08	0.14	0.05	–0.09

Tab. 2. Mean values of total periphytic chlorophyll, water chlorophyll *a* and total water chlorophyll measured in October 2007 and January 2008. The range values (minimum – maximum) for each sample are represented in brackets. Chl – chlorophyll, S – superficial water layer, D – deep water layer

Sampling point	Water depth (m)	Total periphytic chl (μg/cm^2)		Water chl *a* (μg/L)		Total water chl (μg/L)	
October 2007							
E-3S	0.20	2.80	(0.88–4.72)	1.47	(1.26–1.90)	2.35	(1.31–2.45)
E-3D	1.00	0.23	(0.20–0.26)	1.83	(1.48–2.31)	3.34	(1.75–4.49)
E-5S	0.20	2.63	(1.89–3.39)	2.00	(1.70–2.32)	3.57	(2.18–4.15)
E-5D	7.00	0.27	(0.22–0.32)	2.21	(2.14–2.24)	3.82	(2.82–3.21)
E-7S	0.20	7.07	(4.88–9.25)	2.19	(2.12–2.26)	4.14	(0.00–3.16)
E-7D	3.00	–	–	2.25	(2.04–2.36)	3.79	(2.44–3.26)
E-9S	0.20	0.40	(0.28–0.51)	3.19	(3.07–3.38)	5.12	(3.54–4.48)
E-9D	4.00	0.28	(0.21–0.36)	2.69	(2.72–2.73)	4.19	(3.01–3.62)
E-11S	0.20	7.88	(4.61–11.15)	3.42	(3.30–3.57)	5.23	(3.75–5.58)
E-11D	6.00	–	–	3.23	(3.19–3.31)	4.66	(3.83–4.07)
E-13S	0.20	–	–	2.65	(2.16–3.42)	4.99	(2.49–6.42)
E-13D	7.00	1.95	(1.35–2.56)	5.13	(4.90–5.24)	7.65	(5.97–6.43)
E-14S	0.20	–	–	2.40	(1.92–2.67)	5.28	(2.39–5.42)
E-14D	6.80	–	–	5.41	(5.22–5.67)	8.32	(6.51–7.33)
E-15S	0.20	–	–	2.62	(2.46–2.79)	4.71	(3.35–4.46)
E-15D	4.50	0.85	(0.77–0.93)	3.79	(3.65–4.07)	5.71	(4.39–5.53)
January 2008							
E-3S	0.20	5.87	(5.40–6.33)	1.09	(0.81–1.31)	1.69	(0.87–2.93)
E-3D	3.00	0.24	–	1.37	(0.92–2.07)	1.43	(0.84–2.20)
E-5S	0.20	–	–	1.08	(0.68–1.55)	1.59	(0.74–2.80)
E-5D	7.00	–	–	1.10	(1.04–1.24)	1.24	(1.03–1.65)
E-7S	0.20	–	–	0.87	(0.70–1.00)	1.10	(0.70–1.77)
E-7D	3.00	–	–	0.79	(0.06–1.19)	2.13	(1.60–2.66)
E-9S	0.20	–	–	2.44	(2.16–2.69)	3.19	(2.49–3.64)
E-9D	5.00	–	–	1.17	(1.02–1.33)	1.91	(1.44–2.37)
E-11S	0.20	3.72	(2.04–5.40)	6.21	(6.00–6.44)	7.87	(7.61–8.13)
E-11D	6.00	0.55	(0.45–0.65)	1.43	(0.90–2.26)	1.84	(1.17–2.87)
E-13S	0.20	8.71	–	4.32	(4.16–4.52)	5.48	(5.09–5.74)
E-13D	7.00	0.68	(0.64–0.71)	1.78	(0.80–3.43)	2.75	(0.79–5.87)
E-14S	0.20	2.16	–	4.34	(4.00–4.65)	5.11	(4.61–5.48)
E-14D	8.00	–	–	1.55	(1.02–2.09)	2.85	(1.44–4.27)
E-15S	0.20	–	–	3.40	(3.28–3.51)	4.14	(4.09–4.19)
E-15D	4.00	–	–	1.14	(0.70–1.37)	1.39	(0.70–1.44)

(20), followed by *Nitzschia* (13). The 45 diatom taxa with a relative abundance > 0.2% and present in more than 10% of the samples are listed in table 3. Species richness and Shannon-Wiener diversity Index for natural and artificial samples are shown in table 4. No major differences were observed in diatom community diversity between substrata type.

Tab. 3. Diatom taxa of all samples (ranged alphabetically) with relative abundance (RA) > 0.2% and present in more than 10% of the Ebro Estuary samples.

Diatom taxa	% RA	Diatom taxa	% RA
Achnanthes amoena Hustedt	1.29	Navicula recens (Lange-Bertalot) Lange-Bertalot	2.76
Achnanthes minutissima Kützing	1.26	*Navicula* cf. *subminuscula* Manguin	0.52
Amphora aff. h*elenensis* Giffen	0.35	*Navicula* sp.1	0.57
Amphora inariensis Krammer	0.99	*Navicula tripunctata* (O.F. Müller) Bory	0.36
Amphora lybica Ehrenberg	3.55	*Nitzschia amphibia* Grunow	0.58
Amphora ovalis (Kützing) Kützing	0.37	*Nitzschia constricta* (Kützing) Ralfs	0.41
Amphora pediculus (Kützing) Grunow	6.51	*Nitzschia dissipata* (Kützing) Grunow	3.66
Amphora sp.1	0.45	*Nitzschia filiformis* (W. Smith) Van Heurck	1.63
Bacillaria paradoxa Gmelin	3.75	*Nitzschia* cf. *fonticola* (Grunow) Grunow	0.33
Cocconeis pediculus Ehrenberg	0.47	*Nitzschia* cf. *frustulum* (Kützing) Grunow	4.22
Cocconeis placentula Ehrenberg	23.00	*Nitzschia* cf. *inconspicua* Grunow	2.89
Cyclotella meneghiniana Kützing	0.48	*Nitzschia microcephala* Grunow	0.32
Fallacia sp.1	3.33	*Nitzschia palea* (Kützing) W. Smith	1.26
Gomphonema cf. *Olivaceum* (Hornemann) Brébisson	0.33	*Nitzschia* cf. *palea* (Kützing) W. Smith	1.36
Gomphonema clevei Fricke	0.29	*Nitzschia prolongata* Hustedt	0.67
Melosira varians Agardh	0.32	*Nitzschia* cf. s*ociabilis* Hustedt	0.26
Navicula aff. *mollis* (W. Smith) Cleve	1.01	*Pleurosira laevis* (Ehrenberg) Compère	0.61
Navicula antonii Lange-Bertalot	1.40	*Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot	5.86
Navicula capitatoradiata Germain	0.25	*Stephanodiscus* aff. *alpinus* Hustedt	0.21
Navicula cryptotenella Lange-Bertalot	1.96	*Synedra ulna* Ehrenberg	0.33
Navicula aff. c*ryptotenelloides* Lange-Bertalot	0.50	*Tabularia fasciculata* (Agardh) Williams et Round	6.94
Navicula gregaria Donkin	0.83		
Navicula cf. *margalithii* (Lange-Bertalot)	0.91		
Navicula perminuta Grunow	4.47		

Diatom community changed along the horizontal and vertical gradients (Fig. 3). The diatom community of the natural substrata in October 2007 (Fig. 3A) was dominated by *Cocconeis placentula* Ehrenberg (Figs. 4A, B). *Bacillaria paradoxa* Gmelin (Fig. 4H) and *Tabularia fasciculata* (Agardh) Williams et Round (Figs. 4C, D) also appeared as abundant species in most sites (E-3 to E-13). There was a change in diatom community structure in E-14, *Navicula perminuta* Grunow (Fig. 4N) being the dominant species and *Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot (Figs. 4F, G) abundant. Another change occurred in E-15, where *Fallacia* cf. *clepsidroides* Witkowski (Fig. 4J) and *Nitzschia* cf. *inconspicua* Grunow (Fig. 4K) dominated the sample. In January 2008 (Fig. 3B), the dia-

Tab. 4. Species richness and Shannon–Wiener Index for natural and artificial samples in October 2007 and January 2008.

October 2007	Species richness	Shannon–Wiener Index
Natural samples	42	3.03
Artificial samples	34	3.22
January 2008	**Species richness**	**Shannon–Wiener Index**
Natural samples	40	3.61
Artificial samples	42	3.73

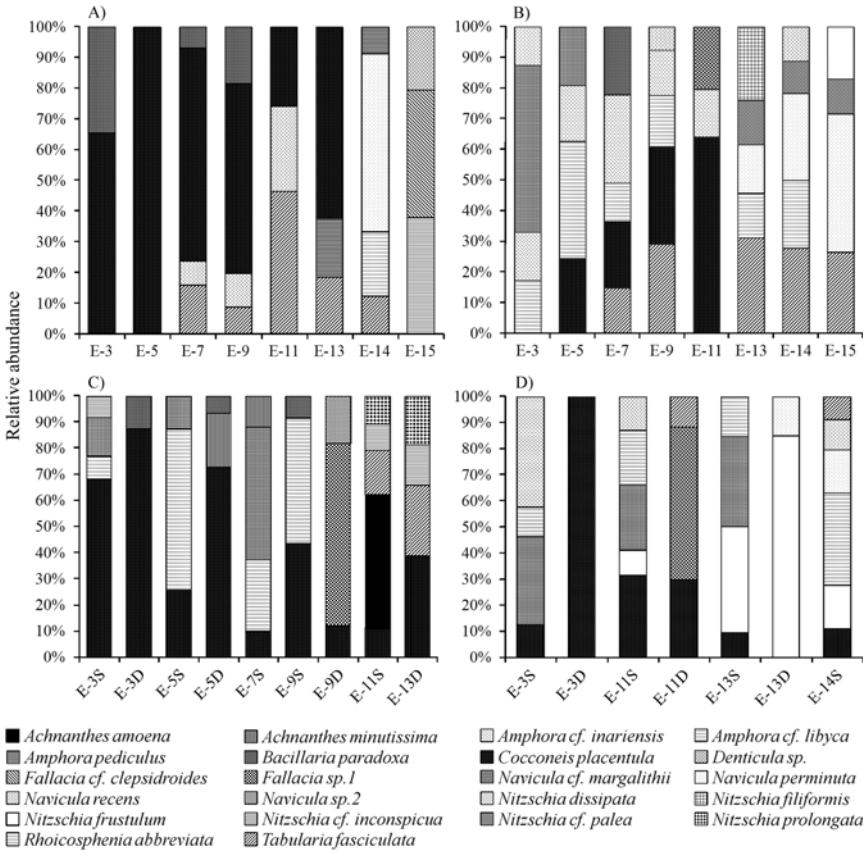

Fig. 3. Diatom community of the Ebro Estuary. A) natural substrata in October 2007, B) natural substrata in January 2008, C) artificial substrata in October 2007, D) artificial substrata in January 2008. Natural substrata were only collected at superficial level. S – superficial water layer, and D – deep water layer. Only diatom taxa with a relative abundance > 0.1 are shown

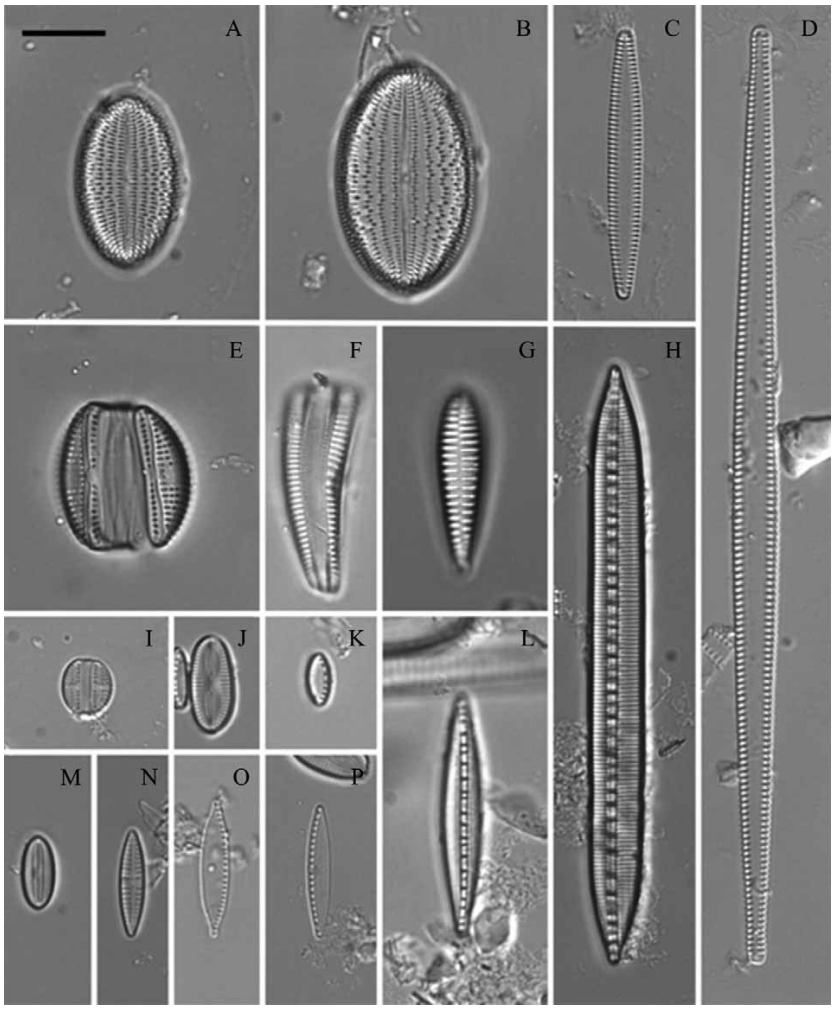

Fig. 4. Representative diatom taxa from the Ebro Estuary. **A, B**: – *Cocconeis placentula* Ehrenberg; **C, D** – *Tabularia fasciculata* (Agardh) Williams et Round; **E** – *Amphora libyca* Ehrenberg; **F, G** – *Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot; **H** – *Bacillaria paradoxa* Gmelin; **I** – *Amphora pediculus* (Kützing) Grunow; **J** – *Fallacia* cf. *clepsidroides* Witkowski; **K** – *Nitzschia* cf. *inconspicua* Grunow; **L** – *Nitzschia dissipata* (Kützing) Grunow; **M** – *Fallacia* sp.1; **N** – *Navicula perminuta* Grunow; **O** – *Nitzschia* cf. *palea* (Kützing) W. Smith; **P** – *Nitzschia* cf. *frustulum* (Kützing) Grunow. Scale bar denotes 10 µm.

tom community was more diverse than in October. In this period a change in the diatom community along the estuary was also observed. In E-13, *Cocconeis placentula* and *Nitzschia dissipata* (Kützing) Grunow (Fig. 4L) drastically decreased and other diatom

taxa became representative, like *Navicula perminuta*, *Nitzschia* cf. *palea* (Kützing) W. Smith (Fig. 4O) and *Nitzschia* cf. *frustulum* (Kützing) Grunow (Fig. 4P), among others.

The diatom community of the artificial substrata in October (Fig. 3C), showed a clear change in E-9D (the limit of the salt wedge), where C*occoneis placentula*, *Amphora libyca* Ehrenberg (Fig. 4E) and *Amphora pediculus* (Kützing) Grunow (Fig. 4I) decreased; and *Fallacia* sp.1 (Fig. 4M) clearly dominated the sample. Unfortunately, several artificial substrata could not be recovered in the January campaign and thus only few data were available (Fig. 3D).

Factors affecting diatom distribution

Results of the correspondence analysis (CA) show that the first dimension of the CA (DIM1) explained 24.6% of diatom community variability, and seems to differentiate the upstream points (with no salt wedge) from those closer to the sea (Fig. 5). This dimension was significantly and negatively correlated with phosphorous (TDP) and positively with water velocity and salinity (Tab. 5). The second CA dimension (DIM2) explained 13.5% of variation and differentiated E-9O (deep layer, October) from the rest of samples. It was significantly and positively correlated with salinity and water temperature; and negatively with oxygen (DO_2), and nitrogen ($N\text{-}NO_3^-$ and TDN).

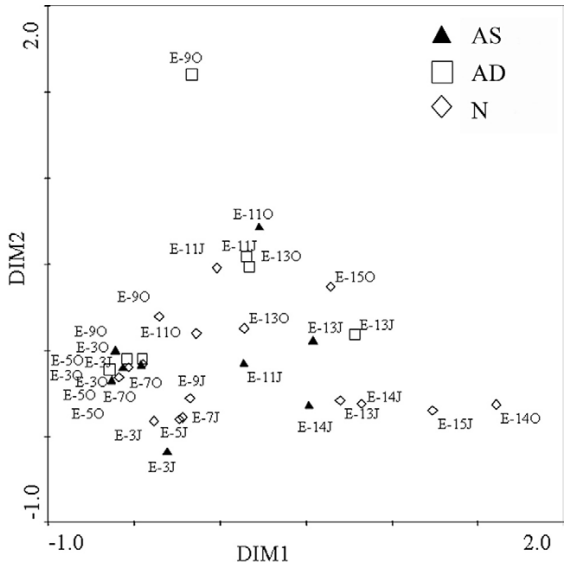

Fig. 5. Sample ordination in the plane defined by the two first CA dimensions. O = October, J = January. Only diatom taxa with a relative abundance (**RA**) > 0.2% and present in more than 10% of the samples are included in the analysis. **AS** – artificial superficial samples, **AD** – artificial deep samples, **N** – natural samples (only collected at superficial level).

Tab. 5. Pearson correlation coefficients between CA dimensions and environmental parameters. Only significant correlations at $P < 0.05$ and with a Pearson correlation coefficient higher than 0.4 are listed.

Environmental parameters	DIM 1	DIM 2
% explained variance	24.6	13.5
Salinity (ppt)	0.431	0.539
TDP (mg L)	–0.465	–
DO_2 (mg L)	–	–0.601
$N\text{-}NO_3^-$ (mg L)	–	–0.609
TDN (mg L)	–	–0.510
Water velocity (m s^{-1})	0.477	–
Water temperature (°C)	–	0.404

Discussion

Water chlorophyll values found in this study were lower than those reported in previous Ebro Estuary papers. The minimum total chlorophyll ($a + b + c$) values in July 1991 were 21.5 µg L^{-1} in the freshwater layer and 3.2 µg L^{-1} in the salt wedge (IBÁÑEZ et al. 1995). From 1989 to 1992 in the freshwater layers the minimum chlorophyll *a* values were 20 µg L^{-1} and 7 µg L^{-1} in the salt wedge (CASAMAYOR et al. 2001); and in July 1999 the minimum chlorophyll *a* values were around 9 µg L^{-1} in the freshwater layer and 2 µg L^{-1} in the salt wedge (FALCÓ et al. 2006). This decrease in phytoplankton has been attributed to a decrease in riverine phosphorous (IBÁÑEZ et al. 2008), which has allowed light to reach the salt wedge and probably has permitted periphytic communities to become established. In July 1989, the light intensity was practically zero at 4.8 m depth, below the interface (CASAMAYOR et al. 2001). In our study, the light reached the river bed (6 m depth in October and 7 m depth in January). In addition, light also reached the river bed in April 2008 (10 m) and July 2008 (7 m), thus the increase in water transparency was not a sampling period effect. However, due to the lower light intensity, the periphytic total chlorophyll concentrations in deep substrata were considerably lower than those found in superficial substrata.

This study allowed identification of the importance in terms of abundance of some periphytic diatom species in the Ebro Estuary, like *Cocconeis placentula,* and the need to study it at infraespecific level in further studies, because different varieties may have different ecological responses. Some of the most representative diatom species found in the Ebro estuary (*Cocconeis placentula* in freshwater layers, *Navicula perminuta* in saline layers, *Amphora pediculus* or *Rhoicosphenia abbreviata*) are not found in the same proportion and/or occurrence in other estuarine studies (MCINTIRE and OVERTON 1971, MOORE and MCINTIRE 1977, MCINTIRE 1978, UNDERWOOD 1994, NAYAR 2005). The Ebro Estuary has specific characteristics (high stratification, irregular and sudden salt wedge intrusions) that are not met in other estuarine systems studied (e.g. Atlantic estuaries, fiords), and these differences could explain differences in diatom flora.

The diatom community structure changed along the Ebro Estuary. A change in diatom community structure was observed in points closer to the sea (from E-13 to E-15) with higher salinity. In these points the marine and riverine influences can be both strong and

they change depending on the river flow, sea storms and winds, resulting in a more dynamic situation at superficial layers. Fluctuating conditions have been previously reported as an important factor affecting diatom community structure, diversity and composition of such environments (SULLIVAN 1978, UNDERWOOD 1994, TROBAJO et al. 2004).

Another important change in the diatom community was found in E-9D in October. This point was the limit of the salt wedge, the interface between fresh and saline water. In this zone the water could remain still at the bottom of the river for a long period. It may well be that the decomposition of organic matter promoted the oxygen depletion found in October. However, the observed oxygen depletion did not reach anoxic conditions that were previously recorded (IBÁÑEZ et al. 1995, CASAMAYOR et al. 2001, FALCÓ et al. 2006). In January, due to the lower river flow, the limit of the salt wedge had to be further upstream (between E-7 and E-9), and unfortunately it was not sampled.

Artificial substrata should allow the comparison of diatom communities from superficial and deep layers, avoiding substratum variability. Several investigations had used artificial substrata to study periphytic diatom communities in estuaries and other transitional systems (MCINTIRE and OVERTON 1971; MCINTIRE 1973, 1978; MOORE and MCINTIRE 1977; LAI 2001; TROBAJO et al. 2004; NAYAR et al. 2005). The CA analysis did not clearly segregate the samples according to the type of substrata (natural vs. artificial). Therefore, it seems that the periphytic diatom community in the Ebro Estuary is more affected by the environmental conditions (mainly salinity, dissolved oxygen, nutrient concentrations) than by the substrata type. Similar results were found by SNOEIJS (1994) in the study of epiphytes from the Baltic Sea, indicating that epiphytic diatom community composition was more affected by environmental parameters than by macroalgal hosts.

The preliminary hypothesis would indicate that diatom community in the Ebro Estuary is determined by salt wedge intrusions, which cause high and irregular fluctuations of salinity and nutrient concentrations. The diatom community could be also affected by the water residence period, which could cause the oxygen decrease observed from the river mouth to the limit of the salt wedge. These initial results suggest that the factors affecting the diatom community and its distribution in the Ebro Estuary are salinity, phosphorous, nitrogen, oxygen, water temperature and water velocity. The results show a longitudinal variation correlated with salinity and inversely with phosphorous (TDP), and as we expected it could be related to the presence of the salt wedge, which causes a system shift. The highest water residence period was reached in the limit of the salt wedge intrusion. The particular conditions in this site (high salinity, low oxygen and nitrogen depletion) also affected diatom community composition and structure.

Therefore, it seems that the salt wedge dynamics (not only vertical and longitudinal salinity gradients but also the magnitude and frequency of salinity oscillations) have an influence on the periphytic diatom composition of the Ebro Estuary and have to be taken into account in further studies. Estuarine dynamics is different in more mixed estuaries where salinity gradients and salinity changes are more regular and predictable (due to tidal circulation).

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Planothidium iberense sp. nov., a new brackish diatom of the Ebro Estuary, northeast Spain

LAIA ROVIRA[1]*, ANDRZEJ WITKOWSKI[2], ROSA TROBAJO[1], MANFRED RUPPEL[3] & CARLES IBÁÑEZ[1]

[1]*IRTA - Aquatic Ecosystems, St. Carles de la Ràpita, Spain*
[2]*Palaeoceanology Unit, University of Szczecin, Szczecin, Poland*
[3]*Faculty of Biosciences, J.W. Goethe University, Frankfurt am Main, Germany*

This study describes the diatom species *Planothidium iberense* sp. nov. from a salinity gradient of the Ebro River Estuary in northeastern Spain. A detailed description is given based on light and scanning electron microscopy observations, and the diatom species is compared with morphologically similar *Planothidium* species. *Planothidium iberense* is distinguished from *P. linkei* mainly by its morphometric characteristics, but also by the presence of multiseriate striae, in contrast to biseriate striae in *P. linkei*. *Planothidium iberense* is also distinguished from *P. delicatulum* and *P. septentrionale* by the presence of an one-side expanded central area on the sternum valve, which is absent in the latter two *Planothidium* species.

Keywords: *diatom, Ebro, estuary, Mediterranean,* Planothidium*, taxonomy*

Introduction

The genus *Planothidium* was established by Round & Bukhtiyarova (1996) with *Achnanthes lanceolata* (Brébisson) Grunow (=*P. lanceolatum* (Brébisson ex Kützing) Lange-Bertalot) designated as the generitype. Among the most characteristic features of the genus are the bi- to multiseriate transapical striae (areolae barely resolvable in the light microscope, but visible in the scanning electron microscope) on both the raphe valve (RV) and sternum valve (SV). In the SV, the striae can be continuous ('*delicatulum*' type) or interrupted on one side showing a clear space in the central area. This interruption may not be associated with any internal depression ('*minutissimum*' type) or it may be accompanied by a 'hoof-mark' depression ('*frequentissimum*' type when this depression is an internal capped structure; '*lanceolatum*' type where the depression is not capped). The raphe is often centrally expanded at the central valve area, while it is curved at apices.

A new diatom species, *P. iberense* Rovira & Witkowski, is described from the Ebro Estuary. Detailed light (LM) and scanning electron (SEM) microscopy examination of the valve ultrastructure is presented. This new species to science is compared with closely-related species such as *P. delicatulum* (Kützing) Round & Bukhtiyarova and *P. linkei* (Hustedt) Lange-Bertalot.

Materials and methods

The Ebro River is the largest river in Spain, having a delta of 320 km^2 with an actual land use of 40% protected areas (~130 km^2 of Natura 2000 network including the Ebro Delta Natural Park), 55% agriculture (mainly rice fields) and the rest are urban areas. The Ebro Estuary (40° 43′16.59″N, 0°40′37.79″E) is a 32 km stretch of water with a mean depth of 6.8 m and a mean width of 237 m; it covers ~7 km^2. The Ebro Estuary is classified as a Mediterranean salt wedge or highly stratified estuary (Ibáñez et al. 1997). The microtidal range (~20 cm) favors stratification of the water column and the presence of the salt wedge. The river discharge, which is the main factor controlling the hydrological dynamics of this estuary, has been highly regulated by a series of dams built mainly in the mid 1960s.

Benthic diatoms were collected from eight stations along the estuary (distance between stations ~4 km) (Fig. 1). The gradient encompassed both freshwater and saltwater zones of the estuary. Sampling was conducted every three months from October 2007 to December 2008. This work is part of a larger study on the periphytic diatom community in the Ebro Estuary. Although the Ebro Estuary is ecologically and socio-economically important, no previous studies have been conducted on the ecology and taxonomy of its diatom communities. Samples were collected from both natural substratum (mainly macrophytes,

*Corresponding author. Email: laia.rovira@irta.cat

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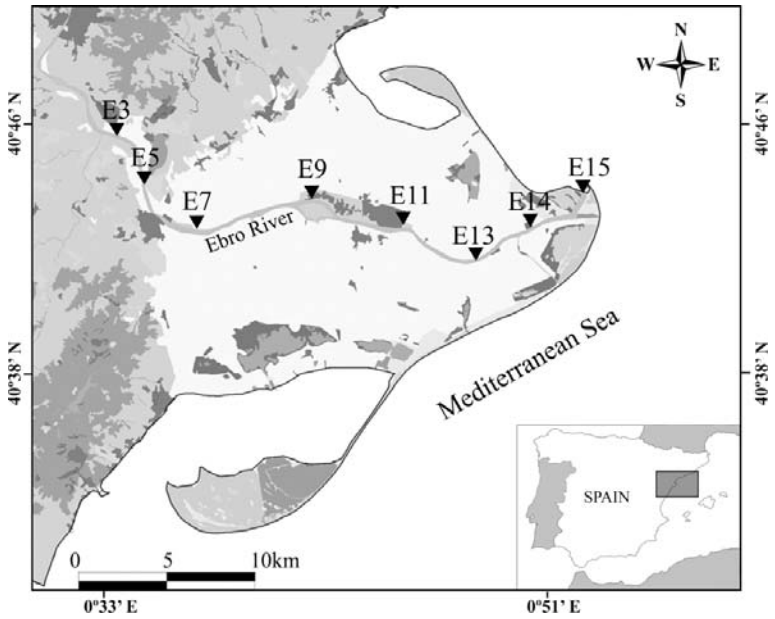


Fig. 1. Position of the eight sampling stations along the Ebro Estuary in northeastern Spain visited from October 2007 to December 2008.

but also wood debris where macrophytes were not available) and from artificial substrata (bricks). Bricks were placed at surface (0.5 m) and deep (2–8 m) water layers. Artificial substrata were used to sample deep water layers and to be able to compare diatom community composition with natural substrata. Two replicates from surface and deep water layers (when they were available) were processed. Two replicates from natural surface substrata were also processed.

Water temperature, electrical conductivity (EC_{25}) and dissolved oxygen (DO_2) were measured in situ for all sampling sites with an YSI 556 multiprobe. Analysis of dissolved inorganic nutrients: silicate (Si-SiO_4^{4-}), nitrate (N-NO_3^-), phosphate (P-PO_4^{3-}); total dissolved nitrogen (TN) and total dissolved phosphorus (TP) were measured following Grasshoff et al. (1999) except for the ammonium (N-NH_4^+) which was measured following the method proposed by the equipment manufacturer (Alliance Instruments, SA).

Diatoms were cleaned of organic matter by boiling the frustules in suspension in 30% H_2O_2 and adding 37% HCl to remove the calcium carbonate. After oxidation, cleaned samples were successively rinsed with deionized water. Permanent microscopic slides were mounted with Naphrax® and examined using a LEICA DMLB with a 100× oil immersion objective (numerical aperture (NA) = 1.40) and Nikon Ecclipse 600 light microscopes equipped with differential interference contrast (DIC) with a 100× oil immersion objective (NA = 1.40) for LM observations. For SEM examination, a drop of the cleaned sample was filtered onto Nuclepore Whatman polycarbonate membranes. Filters were air-dried overnight, mounted onto aluminium stubs, coated with gold–palladium and examined in a Hitachi S-4500 SEM apparatus.

Observations

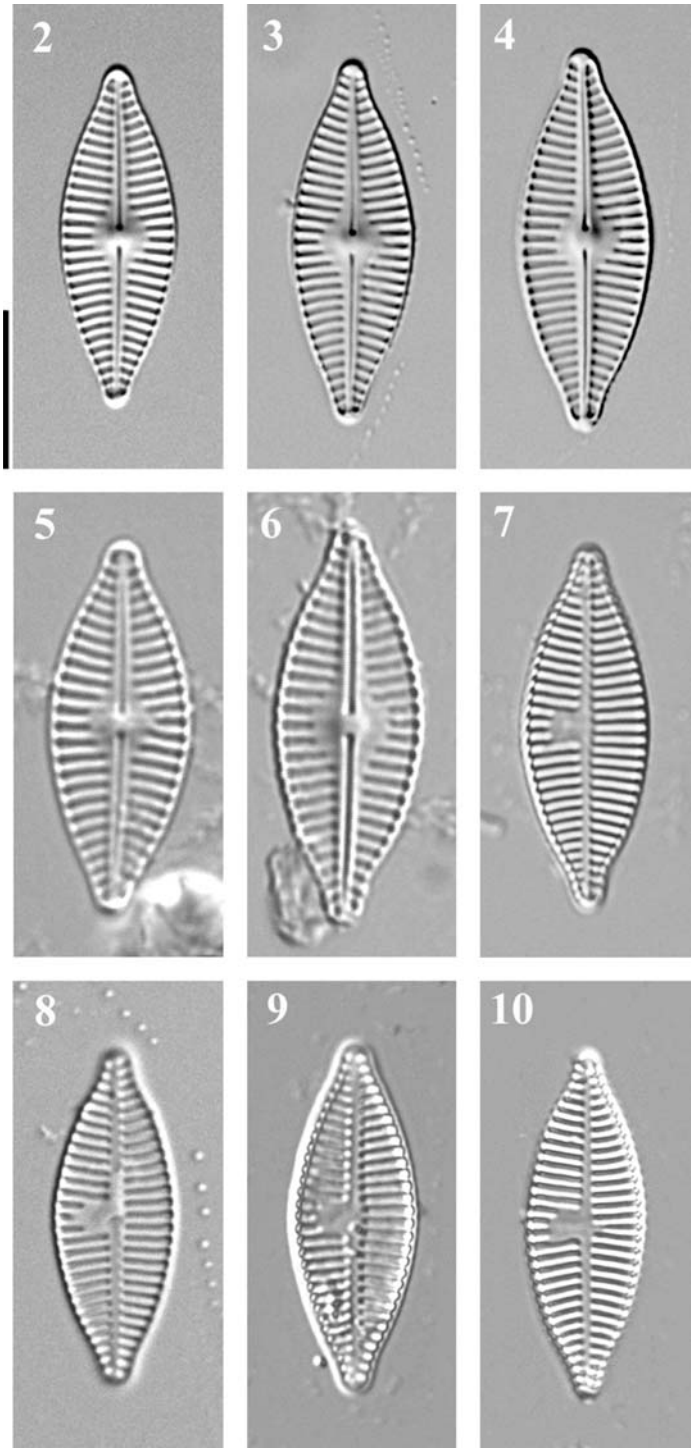
Planothidium iberense Rovira & Witkowski sp. nov.
(Figs 2–24)

Descriptio: *Frustula solitaria rectangulata leviter curvata ad axem transapicalem. Valvae elliptico-lanceolatae apicibus protractis leviter capitatis late rotundatis. Longitudo 17–26 μm, latitudo 6.5–9.5 μm.*

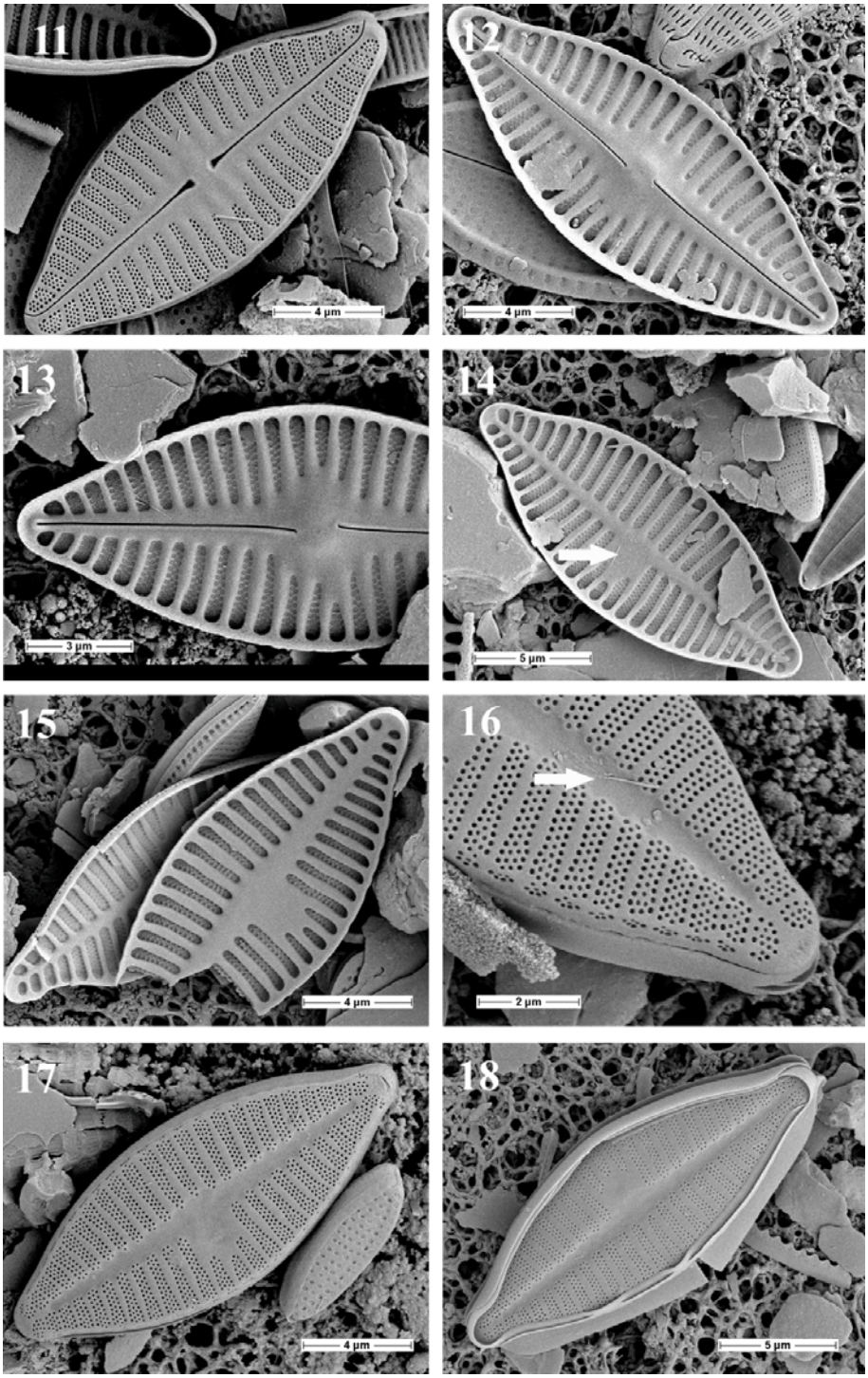
Areovalva. Sternum anguste lineare. Area centralis unilateraliter formata, fere ampla irregulariter rectangulata. Striae transapicales minus radiantes in partibus proximalibus valvae aliquid plus in partibus distalibus, 14–16 in 10 μm.

Raphovalva. Raphe recta, area axialis linearis angustissima prope apices aliquid dilatata versus mediam valvae. Area centralis fere magna circularis. Raphosternum rectum extremis centralibus internis leviter expansis coaxialibus sitis extremisque distalibus non discernendis microscopio photonico. Striae transapicales leviter radiantes proximaliter et aliquid distinctius radiantes prope apices, 12–15 in 10 μm.

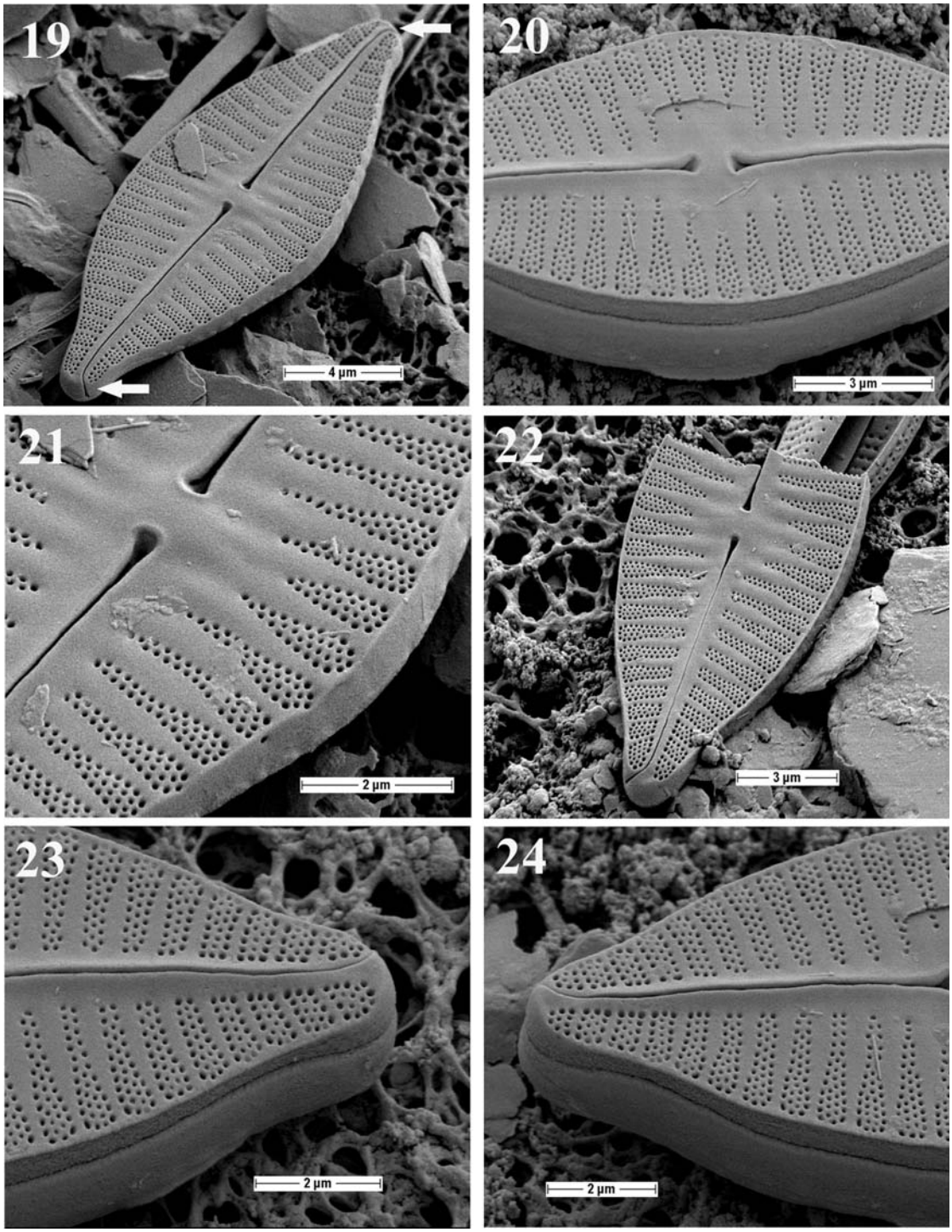
Holotype: Slide no. 14825, collection A. Witkowski, Institute of Marine Sciences, University of Szczecin, Poland.



Figs 2–10. *Planothidium iberense* from the holotype slide 14825, LM. **Fig. 2.** Raphe valve of holotype specimen. **Figs 3–6.** Raphe valve view. **Figs 7–10.** Sternum valve view. Scale bar = 10 μm.



Figs 11–18. *Planothidium iberense*, SEM. **Figs 11–13.** Raphe valve. **Fig. 11.** External view of the raphe valve. **Figs 12–13.** Raphe valve internal view. **Figs 14–18.** Sternum valve view. **Figs 14–15.** Internal view of the sternum valve. Note the presence of unilateral central area (arrow). **Figs 16–18.** Sternum valve external view, note the structure of the striae forming areolae and an increase in number of areolae from 3 to 4 in close to the valve margin, slightly depressed sternum is arrowed in Fig. 16. Note also the extension of striae onto the mantle in Fig. 16 and the shape of the unilateral central area in Fig. 17.



Figs 19–24. *Planothidium iberense* SEM (raphe valve external view). **Figs 19–22.** External central raphe endings and the fine structure of the striae. **Fig. 19.** Note the external central raphe endings and the apical raphe ending (arrows). **Figs 20–21.** Close up of the raphe external central endings. **Figs 23–24.** Close up of the external apical raphe endings, note slightly elevated raphe sternum.

Collected by Laia Rovira on 22 April 2008. Holotype specimen is illustrated in Fig. 2.

Isotype: Slide BM 101398, The National History Museum, London, UK.

Type locality: Ebro Estuary (40 °43′16.59″N, 0 °40′ 37.79″E), on natural and artificial substrata of both surface and deep water layers. Holotype location: E14 deep water layers.

Etymology: The specific epithet refers to the Latin name of the Ebro River (Iberus Flumen).

Description in LM. Frustules solitary, rectangular, slightly bent about transapical axis. Valves elliptical-lanceolate, with broadly rounded, slightly capitate apices, 17–26 μm in length, 6.5–9.5 μm in breadth. RV: raphe straight, axial area linear, very narrow at apices becoming broader towards the valve centre (Figs 2–6). Central area rather large, circular. Raphe-sternum straight, external central raphe endings coaxial, slightly expanded. External apical raphe endings in LM not resolvable. Transapical striae slightly radiate in mid-valve, becoming radiate towards apices, ∼12–15 in 10 μm. SV: linear and narrow sternum. Central area developed only on one side of the sternum, rather large, irregularly rectangular (Figs 7–10). Transapical striae slightly radiate in the central valve part becoming radiate towards the apices, 14–16 in 10 μm.

RV in SEM. Valve face flat, abruptly turning towards the valve mantle (Figs 19–24). In all the specimens observed (n = 15), the striae were not extended onto the mantle (Figs 19–24). Raphe-sternum linear, narrow, slightly elevated, central area circular (Figs 11–13, 19–22). External central raphe endings coaxial, expanded, triangular in shape. External apical raphe endings simply bent to the same side. The transapical striae are multiseriate, composed of four rows of areolae at the valve apices, but changing from four close to the margins to two, even one, near the raphe-sternum (Fig. 11, Figs 19–24). The multiseriate striae are separated by slightly elevated interstriae (Figs 11, 19, 21–22). Internally, the raphe-sternum is slightly elevated with the external central raphe endings bent in opposite directions. Internal apical raphe endings terminate in a small helictoglossa. The striae internally are positioned in shallow depressions separated by elevated, narrow interstriae (Figs 12–13).

SV in SEM. Valve face generally flat rather abruptly turning towards the valve mantle, with a narrow and linear hyaline area at the valve face–mantle junction (Fig. 16). A distinct linear depression indicates the sternum on the external valve face (Figs 16–18), while at centre, there is a distinct, unilateral central area. The transapical striae are multiseriate and composed at the apex of four rows of areolae, varying from four rows at the valve margin

Table 1. Relevant water physico-chemical parameters of sampling sites where *Planothidium iberense* was present.

Station	Water depth (m)	Substrata	Date	Temperature (°C)	DO_2 ($mg\,L^{-1}$)	EC_{25} ($mS\,cm^{-1}$)	$P\text{-}PO_4^{3-}$ ($mg\,L^{-1}$)	TP ($mg\,L^{-1}$)	$N\text{-}NH_4^+$ ($mg\,L^{-1}$)	$N\text{-}NO_3^-$ ($mg\,L^{-1}$)	TN ($mg\,L^{-1}$)	$Si\text{-}SiO_4^{4-}$ ($mg\,L^{-1}$)	RA (%)
E-15	Surface	Natural	January	11.54	13.57	3.55	0.037	0.051	0.049	3.240	3.696	0.660	0.4
E-14	Deep	Artificial	April	14.86	5.57	39.90	0.035	0.032	0.023	1.804	2.576	0.814	18.1
E-15	Surface	Natural	April	15.99	9.69	4.48	0.028	0.052	0.373	3.816	5.075	1.251	16.2
E-15	Surface	Artificial	April	15.99	9.69	4.48	0.028	0.052	0.373	3.816	5.075	1.251	1.6
E-15	Deep	Artificial	April	15.91	8.49	39.86	0.034	0.058	0.164	3.905	5.025	1.228	6.1
E-15	Surface	Natural	July	25.11	8.07	6.99	0.009	0.038	0.104	1.418	2.807	0.741	0.1

Notes: All the sampling campaigns where *P. iberense* was found were carried out in 2008. Sup: superficial; DO_2: dissolved oxygen; EC_{25}: electrical conductivity; $P\text{-}PO_4^{3-}$: phosphate; TP: total phosphorous; $N\text{-}NH_4^+$: ammonium; $N\text{-}NO_3^-$: nitrate; TN: total nitrogen; $Si\text{-}SiO_4^{4-}$: silicate; RA: relative abundance in the sample (n/N), where n is the number of *P. iberense* valves and N is the total number (at least 800) of diatom valves counted in the sample.

Table 2. Morphometric data and morphological features of *Planothidium iberense* compared with other morphologically-related *Planothidium* species.

	P. delicatulum	*P. septentrionale*	*P. linkei*	*P. iberense*
Length (μm)	7–20[1] 10–26[2]	7–20	20–40[3] 26–38[4] 34–38[5]	17–26
Width (μm)	4–8[1] 5–10[2]	4–8	10–14[3] 10–14[4] 12–15[5]	6.5–9.5
Apex	Slightly produced, wedge-shaped, obtusely rounded	Cuneate obtusely to broadly rounded	Strongly acute[3] Markedly protracted[4,5]	Moderately produced, obtusely rounded
Sternum valve				
Striae in 10 μm	14–16[1] 17–19[2]	11–14	11–12[3] 11[4] 11[5]	14–16
Striae arrangement	Multiseriate Parallel in the middle, radiate at apices	Multiseriate Parallel in the middle, radiate at apices	Biseriate, coarse, slightly radial	Multiseriate Parallel in the middle, radiate at apices
Sternum	Narrow, linear	Narrow close to linear	Narrowly lanceolate	Narrow, linear
Central area	Absent	Absent	Unilateral	Unilateral
Raphe valve				
Striae in 10 μm	15[1] 14–16[2]	10–14	11–12	12–15
Striae arrangement	Multiseriate Radiate	Multiseriate Radiate	Biseriate Radiate	Multiseriate
Raphe	Straight	Straight, external central endings moderately distant	Straight	Straight
Axial area	Narrow, linear	Narrow, linear	Very narrowly linear	Narrow, linear
Central area	Small	Moderately large to large, circular	Fairy large elliptical to rectangular	Moderately large

Notes: [1]Round & Bukhtiyarova (1996), [2]Hustedt (1933), [3]Witkowski et al. (2000), [4]Hustedt (1939), [5]Andrews (1981).

to three rows along the sternum; all striae are separated from each other by flat and narrow interstriae (Figs 16–18). The striae extend onto the valve mantle but it is interrupted by the hyaline band at the valve face–mantle junction (Fig. 16). The girdle is composed of a few (at least 2) open bands (Fig. 18). Internally the sternum is slightly elevated whereas the transapical striae are positioned in depressions separated by elevated interstriae (Figs 14–15).

Distribution. Planothidium iberense was found during three sampling campaigns: January 2008, April 2008 and July 2008. It was found in sites closer to the sea, at both the deep water layer (with salt wedge influence) and surface water layer (without salt wedge presence), and on both natural and artificial substrata. Its maximum abundance was recorded in April 2008. The physico-chemical parameters during the sampling period where *P. iberense* was found as well as its relative abundance are presented in Table 1.

Discussion

The new species *Planothidium iberense* exhibits most of the characteristics used to establish *Planothidium* as a new genus in Round & Bukhtiyarova (1996), such as multiseriate striae, the presence of a central region expanded on one side on the SV (belonging to the '*minutissimum*' group, species with an interruption of the striae on the SV and absence of either depressions or raised valve parts), prominent raphe centrally expanded and curved to one side in apices. Interestingly, in the genus description, Round & Bukhtiyarova (1996) mentioned the presence of a rather inconspicuous ring of areolae on the mantle, although such a feature is impossible to distinguish in their illustrations. However, *P. iberense* presents an extension of multiseriate striae onto the mantle in SV forming a 'cluster' of areolae rather than a 'ring', and it is clearly visible under the SEM (Fig. 16). This extension of multistriate striae onto mantle in SV is a common feature in other *Planothidium* species, such as *P. daui* (Foged) Lange-Bertalot, *P. granum* (Hohn & Hellerman) Lange-Bertalot, and *P. minutissimum* Krasske (Morales 2006), but in any of these species the extension of the striae is interrupted by a hyaline band, a character that seems to be unique to *P. iberense*.

Planothidium iberense also shares other characteristics with most *Planothidium* species, for example, a depressed sternum on SV, penetration of the distal ends of the raphe onto the valve mantle, the tear-drop aspect of the proximal raphe ends in external view and their deflection in internal view and the presence of a helictoglossa. However, *P. iberense* also presents other unique ultrastructural features, as the abrupt transition (almost 90°) between valve face and valve mantle and the absence of areolae in the raphe valve mantle.

Planothidium iberense is morphologically close to *P. delicatulum*, *P. septentrionale* and *P. linkei*. All of these taxa have a similar valve outline and striae pattern (Henderson & Reimer 2003). Additionally, all the mentioned taxa occur in brackish–marine habitats (Witkowski et al. 2000).

Planothidium iberense has a comparable valve pattern with *P. linkei*, with capitate apices (although in *P. iberense* the apices are much less distinctly capitate than in *P. linkei*), and with the central area of the SV being unilateral for both species. Despite these similarities, *P. iberense* may be distinguished from *P. linkei* by its morphometric data, the major differences are width and transapical stria density of both the RV and the SV (Table 2). The width of *P. iberense* ranges from 6.5 to 9.5 μm, whereas that of *P. linkei* ranges from 10 to 14 μm. In addition, *P. linkei* possesses biseriate striae resolvable under electron microscope (Andrews 1981, Lange-Bertalot & Krammer 1989, Krammer & Lange-Bertalot 1991), whereas *P. iberense* has multiseriate striae (Figs 11–24).

Planothidium iberense can also be compared with *P. delicatulum*. The two taxa have similar valve outlines, multiseriate striae and their morphometric measures are similar (Table 2). However, in *P. delicatulum* the RV central area is missing, whereas in *P. iberense* it is consequently present. Similar is the comparison with *P. septentrionale* in which the central area is missing in SV (Witkowski et al. 2000).

The distribution of *P. iberense* in the Ebro Estuary seems to be confined to the area closest to the sea, influenced directly by marine waters at deep water layers (due to the salt wedge intrusion), but also (with minor effect) at surface water layers. The highest abundances of *P. iberense* were found in the spring, regardless of salinity differences, water depth and substrata type. At these locations, the diatom community is dominated by several widespread fresh-brackish, brackish and marine diatom species, such as *Nitzschia inconspicua* Grunow, *Nitzschia frustulum* (Kützing) Grunow, *Navicula perminuta* Grunow, *Cocconeis* cf. *neothumensis* Krammer, *Rhoicosphenia abbreviata* (Agardh) Lange-Bertalot and *Tabularia fasciculata* (C. Agardh) Williams & Round (Rovira et al. 2009).

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Paedogamy and auxosporulation in *Nitzschia* sect. *Lanceolatae* (Bacillariophyta)

David G. Mann[1]*, Shinya Sato[1,2], Laia Rovira[3] and Rosa Trobajo[3]

[1]*Royal Botanic Garden Edinburgh, Inverleith Row, Edinburgh EH3 5LR, Scotland, UK*
[2]*Cardiff University, Cardiff CF10 3XQ, Wales, UK*
[3]*Aquatic Ecosystems, Institute for Food and Agricultural Research and Technology (IRTA), Crta de Poble Nou Km 5.5, Sant Carles de la Ràpita, Catalunya, E-43540, Spain*

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Paedogamy (fusion of gametes produced within the same gametangium following meiosis) has rarely been reported in diatoms, with fewer than 10 confirmed examples. One of these, reported by L. Geitler, was in a diatom from Illmitz, Lake Neusiedl (Austria), identified as '*Nitzschia frustulum* var. *perpusilla*'. We observed uniparental auxosporulation in two *Nitzschia* clones isolated from the lower Ebro River (Catalonia, Spain), morphologically similar to Geitler's material and belonging to the *N. inconspicua* species complex. We established that the auxospores were formed paedogamously by Feulgen staining of the nuclei and time-lapse microscopy of living cells. However, reinvestigation of Geitler's original cytological preparations revealed differences between the Illmitz and Ebro material with respect to the length of the initial cells, the structure of the perizonium, and the timing of degeneration of superfluous haploid nuclei during gametogenesis, indicating a genetic and possibly a taxonomic separation. Scanning electron microscopic studies of Ebro auxospores revealed a novel form of longitudinal perizonium with bilateral asymmetry, and also scaly incunabula surrounding the unexpanded zygote, which contrast with the strip incunabula of another paedogamous *Nitzschia* species, *N. fonticola*. Molecular phylogenies, based on *rbc*L and partial LSU rDNA sequences, and evaluation of trees constrained to make the paedogamous species monophyletic, indicate that paedogamy probably evolved at least twice independently in *Nitzschia* sect. *Lanceolatae*, in the *N. inconspicua* and *N. fonticola* lineages.

Key Words: Archived cytological material, Auxosporulation, Cytology, Feulgen stain, Inbreeding, Incunabula, LSU rDNA, *Nitzschia*, Paedogamy, Perizonium, *rbc*L, Sexual reproduction

INTRODUCTION

The sexual phase of the diatom life cycle has received increased attention during the last 20 years, with many new reports and reviews (e.g. Chepurnov *et al*. 2004) adding to the classic literature (for which see Geitler 1932, 1973, 1984; von Stosch *et al*. 1973; Round *et al*., 1990). The new information has produced a few surprises, especially the discovery that heterothally is not uncommon (Roshchin 1994). When Drebes (1977) wrote his review of diatom sexuality, heterothally (dioecy) was known in just one diatom (*Rhabdonema adriaticum* Kützing), most other diatoms studied being homothallic. It seems now that heterothally may in fact be the predominant sexual system in pennate diatoms (review by Chepurnov *et al*. 2004; more recent papers include Mann & Chepurnov 2005; Chepurnov *et al*. 2005; Poulíčková *et al*. 2007; Vanormelingen *et al*. 2008; Trobajo *et al*. 2009; Davidovich *et al*. 2009, 2010; Mann & Poulíčková 2010; Davidovich & Davidovich 2011; Sato *et al*. 2011), although homothally is also not infrequent (Geitler 1932; Poulíčková & Mann 2006; Mann *et al*. 2009; Quijano-Scheggia *et al*. 2009). However, the proportion of species studied is still extremely small, and those that have been investigated may yet prove to be atypical of the majority, just as heterothallism was itself once regarded as the exception. Another possibility is that mating systems vary systematically among families and genera of pennate diatoms, some groups being predominantly heterothallic whereas others are homothallic or automictic, and that our sampling of these is very uneven.

In contrast to homo- and heterothally, automixis (self-fertilization within a single gametangium) currently appears to be uncommon in diatoms. Among more than 90 taxa of pennate diatoms reviewed by Geitler (1973), automixis occurred in only 12. Since 1973, the number of reports of automixis in pennates has grown (Geitler; 1985; Trobajo *et al*. 2006; Edlund & Spaulding 2006; Poulíčková 2008; Poulíčková & Mann 2008; Mann *et al*. 2011), but automicts still represent a small minority of all pennates studied. In centric diatoms, automixis appears to be even rarer, with records only for *Cyclotella meneghiniana* Kützing (Iyengar & Subrahmanyan 1944), *Melosira nummuloides* C. Agardh (Erben 1959) and *Thalassiosira angulata* (Gregory) Hasle (Mills & Kaczmarska 2006).

Automixis is not restricted to particular genera or families. Instead, it seems to occur irregularly in widely separated lineages of diatoms whose other members are allogamous (Geitler 1932, 1973, 1985). Indeed, some examples are known in which populations of a single species behave differently, some being automictic, others allogamous, for example, in *Gomphonema angustatum* (Kützing) Rabenhorst (Geitler 1960, 1970b), *Denticula tenuis* Kützing (Geitler 1953) and *Synedra ulna* (Nitzsch) Ehrenberg (Geitler 1939a, b). At first sight, the pattern of distribution of auto- and apomicts suggests that these are 'evolutionary dead ends' (because

* Corresponding author (d.mann@rbge.org.uk).

these types of reproduction seem to occur sporadically across the diatoms rather than being concentrated in one or a few lineages), but, as with hetero- vs homothally, relevant data are sparse and patchy.

Two variants of automixis exist. In one, termed autogamy, there is no cytokinesis in the gametangium and fertilization involves fusion between two haploid nuclei within the undivided gametangial cell. This is known in *Cymbella ventricosa* (C. Agardh) C. Agardh (Geitler 1958; here and for other diatoms studied by Geitler, we give the names he used), *D. tenuis* (Geitler 1953), *Pinnularia nodosa* (Ehrenberg) W. Smith (Poulíčková & Mann 2008) and some species in the *Sellaphora pupula* (Kützing) Mereschkowsky complex (Mann *et al.* 2011 and unpublished observations). In the second variant – paedogamy – cytokinesis occurs after meiosis I so that two separate gametic cells are produced within a single gametangium, which subsequently fuse. This has been reported in an *Amphora* species (Thaler 1972), *Cymbella aspera* (Ehrenberg) Cleve (Geitler 1956), *G. angustatum* (Geitler 1960, 1970b), *Gomphonema constrictum* var. *capitatum* (Ehrenberg) Grunow (Geitler 1952, 1970c), *Epithemia turgida* (Ehrenberg) Kützing (Geitler 1977), a *Neidium* species (Poulíčková 2008), *Nitzschia frustulum* var. *perpusilla* (Rabenhorst) Grunow (Geitler 1970a) and *Nitzschia fonticola* (Grunow) Grunow in Van Heurck (Trobajo *et al.* 2006). Besides these records of autogamy and paedogamy, which were mostly confirmed by cytological observations and/or nuclear staining with acetocarmine or DAPI, there are some less well documented examples in which auxospores have been seen to develop from unpaired mother cells but where no details about meiosis or gamete formation are available. These cases of 'uniparental auxosporulation' may have been autogamous, paedogamous or perhaps even apomictic; examples include two *Nitzschia* taxa (see Geitler 1932, p. 220), *Synedra vaucheriae* (Kützing) Kützing (Geitler 1958) and possibly '*Navicula minima*' (Granetti 1968).

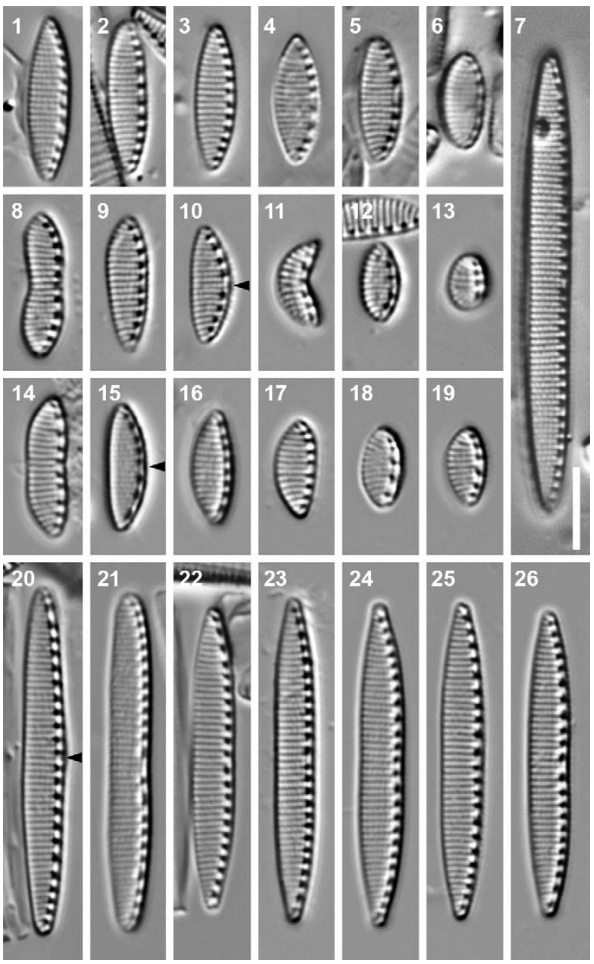
In this article, we focus on paedogamous clones of the *Nitzschia inconspicua* Grunow complex that we isolated from the Ebro River, Catalonia, Spain, and on Geitler's paedogamous Lake Neusiedl material of '*N. frustulum* var. *perpusilla*' (Geitler 1970a), which we discovered has been preserved in the University of Vienna herbarium. Using these materials, we aimed to (1) use morphological data to examine whether the Lake Neusiedl material and the Ebro clones are conspecific; (2) determine whether paedogamy and auxospore structure are identical in the Ebro and Lake Neusiedl populations and in another paedogamous species of *Nitzschia* sect. *Lanceolatae*, *N. fonticola*; and (3) use *rbc*L and partial LSU sequence data to test whether paedogamy has evolved more than once in *Nitzschia*, in the *N. fonticola* and *N. inconspicua* lineages, respectively. *Rbc*L and partial LSU have both proved useful for systematics investigations in *Nitzschia* (Trobajo *et al.* 2006, 2009, 2010) and other diatoms (Mann & Evans 2007; Theriot *et al.* 2010 for *rbc*L), and they have also been recommended for use as diatom barcodes (Mann *et al.* 2010; Hamsher *et al.* 2011). A more detailed examination of evolutionary relationships among clones of *N. inconspicua* and related taxa will be made in a subsequent article (L. Rovira, personal communication).

MATERIAL AND METHODS

Clones 61 and 62 were isolated on 18 June 2010 from stones collected from the River Ebro at Aldover, Catalonia, Spain (40°52′51.80″N, 0°30′24.20″E). *Nitzschia inconspicua* appeared to be abundant in the sample (visual assessment), as in previous samples from the Ebro River (our unpublished data), including its estuary (Rovira *et al.* 2009, 2012a, 2012b). Environmental data on the day of sampling (8 June 2010) were as follows: depth of sample 90 cm, water temperature 21.3°C, conductivity 0.992 mS cm^{-1}, salinity 0.53, and pH 7.95. Given the low salinity, cells were initially isolated into and maintained in WC medium (Guillard & Lorenzen 1972). However, cultures maintained in WC did not prosper: cells looked unhealthy, and some became deformed (probably in part because they were approaching the minimum viable size) and grew in clumps. We therefore tried them in mixtures of freshwater and marine media. Vigorous vegetative growth and paedogamous auxosporulation were obtained with 3:1 and 1:1 mixtures of WC and f/2 medium (for the composition of f/2, see McLachlan 1973; we used seawater from Alfacs bay, off the Ebro Delta, with salinity *c.* 35). The cytological observations reported here, however, were made on material growing in 1:1 mixtures (*c.* 17) of WC and R medium (Chepurnov & Mann 1997), the latter prepared with seawater from the North Sea off Dunbar, Scotland.

Clones 61 and 62 were identified as *N. inconspicua* by reference to the type material of this species, *N. frustulum* (Kützing) Grunow in Cleve & Grunow and similar species, studied by Trobajo & Cox (2006) and Trobajo *et al.* (2012, 2013); they correspond to *N. inconspicua* as illustrated by Krammer & Lange-Bertalot (1988) in their plate 69, figs 1–4 (but not to their figs 6–10, which show a different species, *N. soratensis*: Trobajo *et al.* 2013). Further details concerning the identification of clones 61 and 62 and the taxonomy of *N. inconspicua* are given in the Supplementary Text.

Because of extensive changes to the taxonomy of *Nitzschia* made since 1970 (contrast Hustedt 1930 with Krammer & Lange-Bertalot 1988), it was unclear whether the paedogamous diatom studied by Geitler (1970a) was truly *N. frustulum*. We therefore sought to discover whether any of Geitler's material had survived and could be studied. Enquiries to Dr Walter Till, curator of the University of Vienna herbarium (WU), established that a collection of Geitler's slides still existed there, though its value and condition were unknown. Examination of the collection in summer 2009 (by D.G.M.) revealed that the collection provides vouchers for most of the papers on diatoms written by Geitler from the 1920s on, spanning 50 years (see publication list by Schmid 1991). A brief introduction to the collection is given at http://rbg-web2.rbge.org.uk/algae/collections_Geitler.html. Many of the preparations are in excellent condition, and, although some stains have faded, most of the acetocarmine, safranin and haematoxylin preparations are still bright. Box 15 at WU contains slides made in January 1970 for Geitler's (1970a) paper on '*N. frustulum* var. *perpusilla*' (Supplementary Table 1). Valves of '*N. frustulum* var. *perpusilla*' are illustrated in Figs 1–7, and in the Supplementary Text we discuss the taxonomy of this



Figs 1–26. Cleaned valves, LM. Slight thickenings of the valve margin visible at the centre in some valves (arrowheads in Figs 10, 15 and 20) reflect the presence of central raphe endings. Scale bar = 5 μm.

Figs 1–7. Illmitz material of '*N. frustulum* var. *perpusilla*': Geitler collection, slides 15/28A, 15/28B (Fig. 4) and 15/27A (Fig. 6). Size reduction series of small preauxospore valves (Figs 1–6) and postauxospore valve (Fig. 7).

Figs 8–19. Small, preauxospore valves of *N. inconspicua*, clones 61 (Figs 8–13) and 62 (Figs 14–19). Many cells were deformed, with central constrictions (e.g. Fig. 8), heteropolarity (e.g. Figs 9, 17) or cymbelloid shape (e.g. Figs 10, 15, 16).

Figs 20–26. Enlarged, postauxospore valves of clones 61 (Figs 20–22) and 62 (Figs 23–26).

diatom. For reasons explained there, we refer to Geitler's diatom throughout by the name that he gave it, even though this name is certainly wrong.

According to information in the lid of the slide box (stating '*Nitzschia frustulum* Agar Illmitz 13. 14.I.70') and in Geitler (1970a), all of the slides were prepared from agar cultures. Examination of the slides shows that none of Geitler's cultures were clonal or even unialgal; for example, other species of *Nitzschia* sect. *Lanceolatae* are present in addition to the paedogamous '*N. frustulum* var. *perpusilla*' and also species of *Navicula*, *Gomphonema* and *Caloneis*. It is unclear exactly when the original material for Geitler's cultures was collected in the autumn of 1969, but all came originally from samples among reeds and algae on the shore of Lake Neusiedl at Illmitz, where Geitler states that he found '*N. frustulum* var. *perpusilla*' growing abundantly. Lake Neusiedl has no outflows and a naturally high salinity (for freshwater), with a conductivity of 1.5–3 mS cm^{-1} (*c.* 0.5–1.5) and a high pH of 7.5–10 (Padisak & Dokulil 1994). Geitler (1970a) records that cells grew well on agar made with Bristol or alkaline Knop solution (for the composition of these freshwater media, see Bristol 1919 and Pringsheim 1946, respectively) but remained vegetative. Auxosporulation occurred within a few days of transference of cells to agar composed of ⅛ seawater from the Adriatic, ⅛ soil extract and ¾ distilled water (i.e. a salinity of *c.* 4.8). Hence, the same pretreatment (increase of salinity) promoted auxosporulation in '*N. frustulum* var. *perpusilla*' as in the Ebro *N. inconspicua*.

It seems likely that all the slides preserved in Vienna are from Geitler's cultures (salinity 4.8) rather than from Knop or Bristol agar because almost all are recorded (on the labels or on the lid of box 15) as bearing one or more stages in auxosporulation. As well as stained material, there are incinerations, made to allow examination of frustule morphology. Geitler also examined preparations of live cells, but of course all of these perished long ago.

Microscopy and molecular phylogenetics

For observations of paedogamy in the Ebro clones 61 and 62, we placed 24 × 50 mm coverslips at the bottom of Petri dishes containing culture medium (a 1:1 mixture of WC and R medium) before inoculation from stocks. The coverslips were colonized by cells and could be removed for examination by mounting on drops of medium after careful cleaning of the lower side; preparations were ringed with white Vaseline to prevent evaporation. Preparations remained healthy for several hours.

In order to study the fate of meiotic nuclei in the Ebro clones, material was stained using the Feulgen reagent as follows, according to a modification of the method described by Jong (1997): (1) cells that had colonized coverslips were fixed in a mixture of ethanol and glacial acetic acid (3:1) overnight, then (2) washed with deionized water three times, (3) hydrolysed in 5N HCl for 30 min, and (4) washed in deionized water for 1 h. Subsequently, (5) the coverslip was placed in Feulgen's reagent for 2 h, (6) rinsed with deionized water three times, (7) dehydrated with an ethanol series (50%, 75% and 95%, each for 5 min, and 100% twice, each for 10 min) and finally (8) mounted in Euparal (Agar Scientific, Stansted, UK).

Frustules of the Ebro clones were cleaned by boiling with 70% nitric acid, followed by repeated washing with deionized water and mounting in Naphrax (Brunel Microscopes, Chippenham, Wiltshire, UK) for light microscopy (LM), or on 13-mm-diameter coverslips for field emission scanning electron microscopy (SEM) of perizonium structure. Some material was prepared for SEM by critical point drying to study the delicate early stages of auxospore formation. For this, clones were grown on coverslips and fixed with 2.5% glutaraldehyde for 1 h. Material still attached to the coverslips

was rinsed with PBS three times, postfixed with 1% osmium tetroxide for 1 h, dehydrated through a graded ethanol series and then infiltrated with acetone and finally dried with an Emitech K850 critical point dryer (EM Technologies, Kent, UK). All the procedures for the microscopical preparations were performed at room temperature. For SEM examination, coverslips were attached to aluminium stubs using carbon pads, coated with platinum and examined with a LEO Supra 55VP instrument, usually at 5 kV with a working distance of 4 mm.

Geitler's preparations were either incinerations mounted in Diatopan or preparations fixed with acetic acid–ethanol mixtures, stained in acetocarmine and mounted in euparal (Geitler 1970a, caption to fig. 3). Our observations of cytological preparations and living cells were made with a Zeiss Axio Imager M2 light microscope with a Plan-Apochromat 100× objective (nominal N.A. 1.4, condenser not immersed) and differential interference contrast optics.

For molecular phylogenetic analysis of *rbc*L and D1–D3 region of rDNA (LSU), all the *Nitzschia* species and related sequences were included that were available in GenBank, with new sequences added as detailed in Supplementary Table 2. *Bacillaria paxillifera* (O.F. Müller) Marsson was selected as an out-group by reference to the phylogenies obtained by Theriot *et al.* (2010), Ruck & Theriot (2011) and Rimet *et al.* (2011). Details of PCR cycles and primers for *rbc*L were as given by Jones *et al.* (2005). The D1–D3 hypervariable domain of 28S rDNA was amplified with the following primers: D1R (forward: 5′-ACCCGCTGATTTAAGCATA-3′; Scholin *et al.* 1994) and D3R (reverse: 5′-TCGGAGGGAACCAGC-TACTA-3′; Nunn *et al.* 1996). PCR reaction volumes were 25 µl and contained 10 ng of DNA template, 2.5 µl of 10× NH4 buffer, 2.5 µl of 50 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs, 0.75 µl of 10 µM forward and reverse primers and 1 unit of Taq polymerase. PCR conditions included one initial denaturation of 94°C for 4 min, followed by 35 cycles each consisting of 1 min at 94°C, 40 s at 56°C and 1 min at 72°C and a final elongation step of 7 min at 72°C. The sequencing primers used were those described above (D1R and D3R) plus D2C (reverse: 5′-CCTTGGTCCGTGTTTCAAGA-3′; Scholin *et al.* 1994). PCR products were purified using ExoSAP-IT (USB, Affymetrix, Santa Clara, California, USA). Sequencing was conducted in 10 µl volumes using 0.32 µM of primer, 1 µl of BigDye v3.1 and 2 µl of sequencing reaction buffer (Applied Biosystems, Carlsbad, California, USA). Excess dye-labelled nucleotides were removed using the Performa DTR V3 cleanup system (EdgeBio, Gaithersburg, Maryland, USA), and sequence products were run on an ABI 3730 DNA sequencer (Applied Biosystems). Forward and reverse reads were edited and aligned using Sequencher 4.5 (GeneCodes, Ann Arbor, Michigan, USA).

Sequences of *rbc*L lacked indels and were aligned manually. Sequences of LSU were first aligned with MAFFT 5 (Katoh *et al.* 2005) and the resulting alignment refined manually by reference to its predicted secondary structure (Sato *et al.* 2008); the alignments are available on request from the authors. All identical sequences were excluded from the analysis as redundant, leaving one representative from each genotype. The sequences used for the analyses are listed in Supplementary Table 2. The final data sets consisted of 88 sequences and 1428 base pairs (bp) for *rbc*L and 174 sequences and 787 bp for LSU. RAxML-VI-HPC, v7.2.6 (Stamatakis 2006), was used for maximum likelihood (ML) analyses with the GTRGAMMAI model, which was determined as the most appropriate model of DNA sequence evolution by MrModeltest (Nylander 2004), both with hierarchical likelihood-ratio tests and the Akaike information criterion. Gamma correction values and a proportion of invariable site of each partition were obtained automatically by the program. RAxML conducted a rapid bootstrap analysis and search for the best-scoring ML tree in one single program run. We performed 100 runs for each data set.

For both *rbc*L and LSU data sets, significance tests, using the approximately unbiased (AU) and nonscaled bootstrap probability (NP) tests of CONSEL (Shimodaira & Hasegawa 2001; Shimodaira 2002), were performed under the ML criterion for *a priori* hypotheses, in which monophyly of paedogamous clones and lineages was constrained against the best trees.

RESULTS

When first isolated, clones 61 and 62 had small valves *c*. 3.5–9 µm long and 2.2–2.8 µm wide, with 24.5–30.6 striae and 11–15 fibulae in 10 µm (Figs 8–19, Table 1). Many of the valves were asymmetrical, possibly as a result of the poor growth in WC medium or infrequent subculturing. Some valves were heteropolar (Figs 9, 17), others cymbelloid, resembling *Cymbellonitzschia* (Figs 10, 15, 16; cf. Cocquyt & Jewson 1994); still others had bizarre irregular shapes (Figs 8, 11). A minority were elliptical (Figs 12, 13, 18). After auxosporulation, valves were much more regular in shape (Figs 20–26), with a linear central portion and ends tapering to acute or very slightly rostrate apices. These longer valves (up to 24.5 µm) had 24.5–25.6 striae and 10–13 fibulae in 10 µm; their widths were 2.5–3.2 µm. In both short and long valves, the central two fibulae were sometimes more widely separated than the others (Figs 10, 12, 21, 23, 26). Where the fibulae were not more widely separated, a tiny thickening could usually be seen (Figs 15, 20, arrowheads), demonstrating the presence of a central nodule (i.e. the raphe is interrupted at the centre), but in some valves even this feature was undetectable. The striae were often visibly punctate when the microscope optics were optimized (Figs 8–26).

Geitler's Illmitz material of '*N. frustulum* var. *perpusilla*' (Figs 1–7) was similar to clones 61 and 62, except that we found no valves smaller than 6.5 µm (Geitler 1970a recorded valves of 5.5 µm). Small valves (6.5–10 µm long, 2.6–3.2 µm wide) had regular, linear-elliptical to elliptical outlines; whereas, the longest valves (e.g. Fig. 7) were linear and over 30 µm long (32.5 µm in our data set; Geitler gave 32 µm). Geitler recorded stria densities of 23–24 in 10 µm ('in kleinsten Zellen auch 25': even 25 in the smallest cells), but our measurements (directly comparable to those for clones 61 and 62) gave 24.5–27, rising to over 30 in 10 µm in the smallest valves; the fibula density was 11–13.5 in 10 µm. The conflict between our stria densities and Geitler's may reflect differences in the method of measurement: we measured along the apical axis of the valve, whereas Geitler may have

Table 1. Selected morphometric data for *N. inconspicua* clones 61 and 62 and Geitler's Illmitz material of '*N. frustulum* var. *perpusilla*'.

	Clone 61	Clone 62	Illmitz
Lengths of gametangia	6.53 ± 1.16 (4.0–9.0, $n = 50$)	6.68 ± 0.83 (4.5–8.0, $n = 49$)	8.65 ± 0.83 (6.5–10.0, $n = 40$)
Lengths of initial cells	20.79 ± 1.70 (17–24.5, $n = 50$)[1]	21.23 ± 2.69 (10.5–23.0, $n = 50$)[2]	29.85 ± 1.34 (26.5–32.5, $n = 40$)
Widths of initial or postinitial valves	2.79 ± 0.26 (2.5–3.2, $n = 6$)	2.83 ± 0.13 (2.6–3.0, $n = 12$)	2.79 ± 0.26 (2.5–3.2, $n = 3$)
Striation density of initial or postinitial valves	25.23 ± 0.36 (24.8–25.6, $n = 6$)	24.83 ± 0.32 (24.5–25.4, $n = 12$)	26.09 ± 0.53 (24.5–27.0, $n = 7$)
Striation density of small valves/gametangia	27.41 ± 1.45 (24.5–29.8, $n = 15$)	27.44 ± 1.38 (25.1–30.6, $n = 15$)	28.61 ± 0.86 (27.6–30.4, $n = 11$)

[1] Nine of these initial cells were clearly stunted. When these were excluded from analysis, the lengths of clone 61 initial cells were 21.21 ± 0.43 μm (17.5–24.5 μm, $n = 41$).
[2] The extremely small initial cell of 10.5 μm long was highly deformed. Without this and seven other outlier, deformed initial cells, the lengths of clone 62 initial cells were 22.30 ± 0.43 μm (21.5–23, $n = 42$).

measured along the valve margin (cf. Anonymous 1975; Geitler did not record how he made his measurements). Our measurements of stria densities in small valves and gametangia of the Illmitz material were slightly higher than those of the two Ebro clones (Table 1), but the difference was not significant ($P > 0.05$, Tukey *post hoc* test).

The valve ultrastructure of *N. inconspicua*, including clones 61 and 62, will be described in more detail elsewhere to accompany a molecular phylogenetic study of the *N. inconspicua* complex (L. Rovira, personal communication) and agrees with the type material of the species (Trobajo *et al*. 2013), but some aspects can be seen in Figs 78 and 80. The stria pores (areolae) are simple, possessing hymenes but lacking cribra, and the striae do not become biseriate within the raphe canal; instead, each transapical stria is represented within the raphe canal by a single round poroid. The raphe is interrupted centrally, with simply undeflected endings.

Paedogamy in *N. inconspicua*

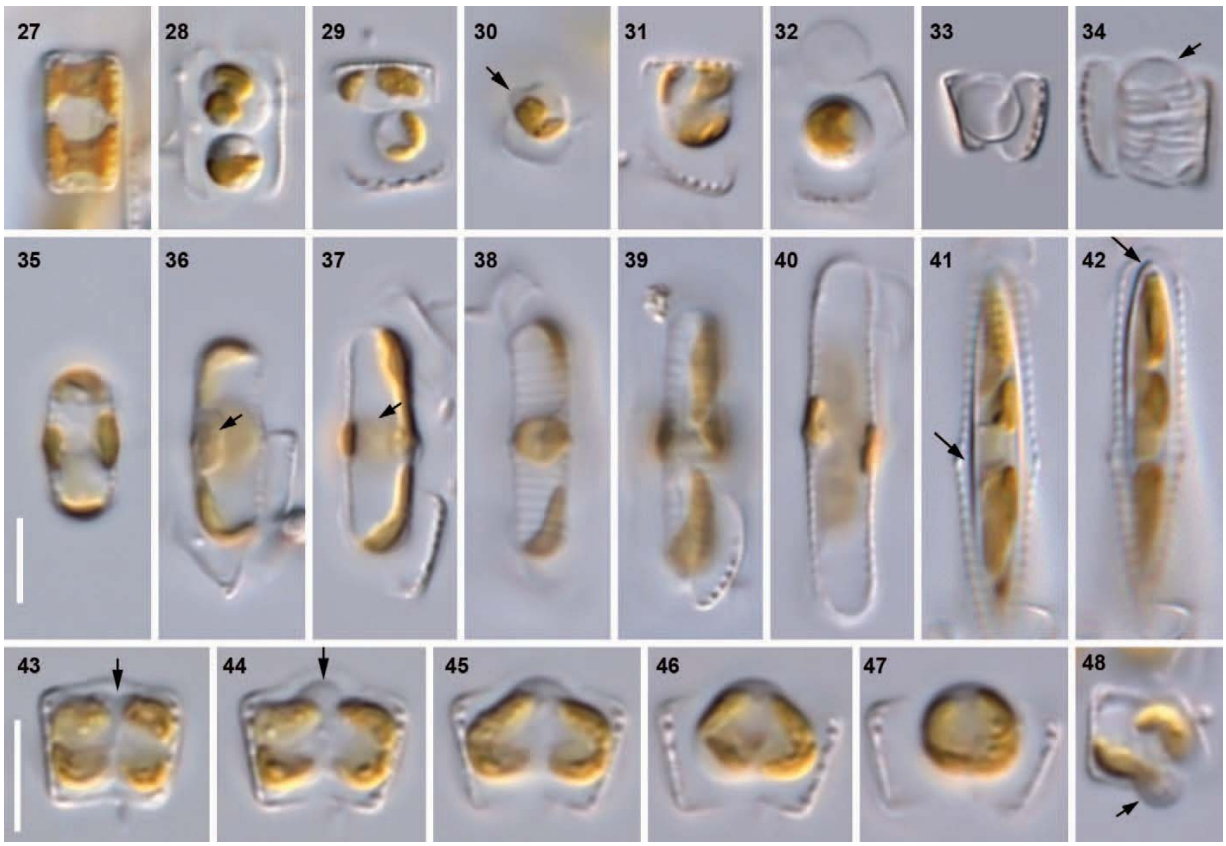
At the time of writing (March 2012), cultures derived from clones 61 and 62 have been cultivated for 1.75 years, and each has declined in size, auxosporulated and declined in size again during several months. There has been no obvious sign of a loss of vitality (manifested in either a reduced growth rate or an increase in the frequency of deformed cells) in the inbred F1 generation.

All instances of auxosporulation within both clonal cultures involved production of a single auxospore from a single parent cell. At times, almost all the cells in a Petri dish (numbering many thousands) were auxosporulating, and no example was observed in which cells paired and exchanged gametes. Mixtures of clones 61 and 62 were made, as were mixtures of these clones with others belonging to the same LSU–*rbc*L clade (our unpublished observations), and again no example was found of interclonal pairing or allogamous sexual reproduction. It is likely, therefore, that paedogamy, as described below, was obligate for clones 61 and 62.

In living cells, the first obvious sign that auxosporulation was imminent was an increase in the width of the girdle to *c*. 5 μm (Fig. 27), compared to ≤ 4 μm for cells in mitotic interphase (not illustrated). Differentiating cells sometimes lay close to other such cells, but they were never paired girdle to girdle or in any other arrangement indicative of allogamous sexual reproduction (e.g. Round *et al*. 1990, figs 59–62; Mann 2011, fig. 3). Subsequently, such cells divided producing two protoplasts (Figs 28, 29), each with two chloroplasts (Fig. 29). In many cells, the protoplasts were rounded and lay one towards each pole (Fig. 28). In other cells, one (Fig. 29) or both protoplasts (Fig. 43, Supplementary Fig. 1A) were less rounded and lay on either side of the median valvar plane, like sibling cells from mitotic division but without the formation of new valves. It seems most likely that division is always in the median valvar plane (cf. Fig. 43) and that the configuration shown in Fig. 28 results from rearrangement after cell division; however, rearrangement was not observed directly.

During or after cytokinesis, the parent cell dehisced and its thecae separated (Figs 28, 29, 44), usually more at one pole than the other (Fig. 44). Next, the protoplasts – now functioning as gametes – fused with each other to form a zygote (Figs 43–47), which was sometimes attached initially to one (Fig. 31) or both (Fig. 45) of the thecae of the parent cell. Soon, however, the zygote separated from the thecae (Figs 32, 47). Plasmogamy was followed *in vivo* in a cell in which the thecae lay more or less at right angles to each other, one lying in valve view and the other in end view (i.e. with the long axis vertical; Supplementary Fig. 1F–J). The protoplast of the 'vertical' theca lost its association with the theca (Supplementary Fig. 1I), moved out and then rounded off at the mouth of the open theca (Supplementary Fig. 1J), by which time it had fused with its sibling protoplast. In other cells whose development was followed *in vivo*, one of the protoplasts was observed to produce a small protrusion prior to plasmogamy (Figs 30, 48), which seemed in one case to function as a pseudopodium, leading the rest of the gamete around its partner (Supplementary Fig. 1C–E).

After plasmogamy, the zygote remained spherical, apparently for some hours (not measured directly but assessed from the frequency with which this stage was observed in clonal cultures), and then expanded bipolarly. The number of chloroplasts was difficult to judge in the contracted, spherical zygotes (Figs 31, 32), but young auxospores had four chloroplasts (Fig. 35), indicating persistence of all four chloroplasts inherited from the gametes. During expansion of the auxospores, the chloroplasts adopted characteristic positions, two being placed on either side of the cell at the centre (Figs 35, 37, 38), whereas the other two lay along the cell, nearer the poles (Figs 36–39): together, they formed a cross (Fig. 39). The centre of the auxospore was occupied by a large vacuole (Figs 35–37, 40), with the nuclei (see next section) lying in a pocket of cytoplasm on one side at the centre (Fig. 36, arrow).



Figs 27–48. *N. inconspicua*, Ebro clones 61 (Figs 28, 39, 43–48) and 62: living cells and acid-cleaned preparations (Figs 33, 34, 41, 42), LM. Scale bars = 5 μm (for Figs 27–42, see bar in Fig. 35; for Figs 43–48, see bar in Fig. 43).

Fig. 27. Gametangium in meiotic prophase.

Fig. 28. Rounded and rearranged gametes within dehisced gametangium.

Fig. 29. Gametangium with unrearranged gametes, one remaining attached to and enclosed within one of the parental thecae, the other rounded up.

Fig. 30. A single gamete, bearing a small protuberance (arrow).

Fig. 31. Young zygote, still attached to one of the parental thecae.

Fig. 32. Zygote.

Fig. 33 Acid-cleaned preparation at a stage equivalent to Fig. 32: note that the zygote wall has survived oxidation.

Fig. 34. Acid-cleaned preparation of an auxospore during the early phase of expansion (± equivalent to the stage in Fig. 35): the zygote wall has been split into two hemispherical caps (arrow) separated by the first bands of the perizonium.

Figs 35–39. Stages in auxospore expansion (Figs 37 and 38 show different focuses of the same cell). Four chloroplasts are present, arranged in a characteristic pattern, two at each end and two on opposite sides at the centre (Figs 35 and 39). The unfused nuclei lie in a pocket of cytoplasm on one side at the centre of the auxospore (Fig. 36, arrow), most of the cell lumen being occupied by a large vacuole (arrow, Fig. 37). The transverse perizonial bands can be seen in surface focuses (Fig. 38).

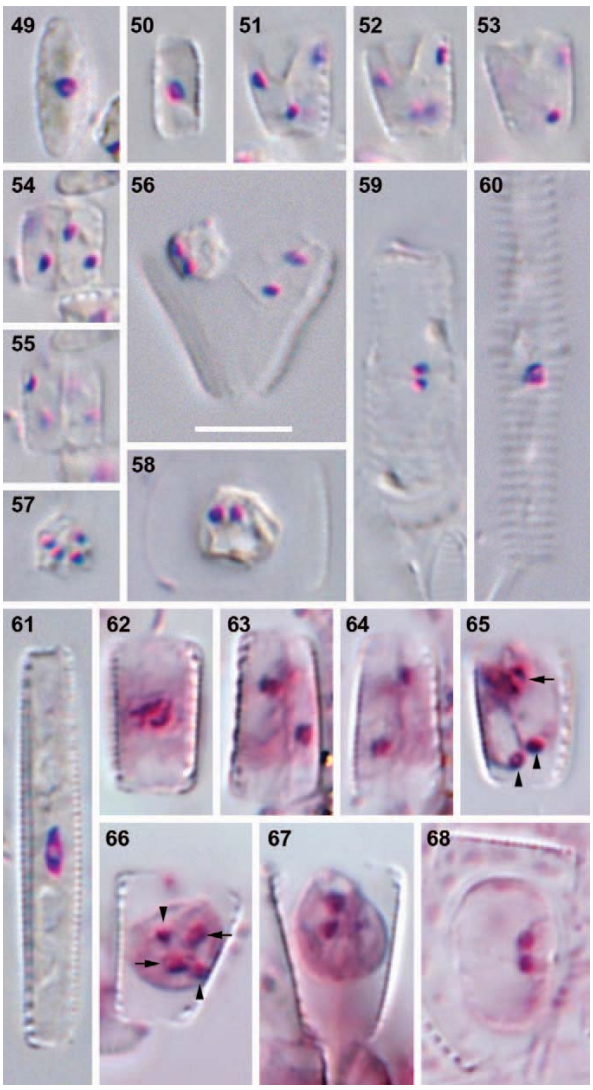
Fig. 40. Fully expanded auxospore in optical section, showing the parietal chloroplasts and large central vacuole.

Figs 41, 42. Initial cell in valve view, still enclosed within the perizonium: two focuses of the same cell. Note that the initial cell does not fill the perizonium, especially at the centre and poles (arrows), as a result of a contraction of the protoplast before formation of the initial epitheca and hypotheca.

Figs 43–47. Plasmogamy in a single gametangium observed at times 0, 61, 80, 97 and 155 min. In Figs 43 and 44, the gametes are still separate (along the midline arrowed in Fig. 43), whereas in Figs 45–47, they have fused to give a zygote, which contracts away from the parental thecae and rounds off. The left-hand gamete extends a small protrusion (Fig. 44, arrow) across the top of the right-hand gamete before plasmogamy.

Fig. 48. Dehisced gametangium containing an apparently amoeboid gamete, which has formed a pseudopodium-like protrusion (arrow; see also Supplementary Fig. 1, C–E).

In *N. inconspicua* cells, the nuclei stained intensely with the Feulgen reagent (Figs 49–61). Interphase vegetative cells contained a central nucleus (Figs 49, 50), in which a single paler nucleolus was usually visible (Fig. 49, 61). Cells that had undergone meiosis contained two protoplasts, each with two equal nuclei (Figs 54, 55). All four haploid nuclei from meiosis were still visible in gametangia in which the frustules had dehisced and the gametes were beginning to separate (Figs 51–53) and also in gametangia containing fully separate rounded gametes (Fig. 56, equivalent to the stage shown in Fig. 28).



Figs 49–68. Nuclear changes during paedogamy: LM of stained material. Scale bar = 5 μm.

Figs 49–61. *N. inconspicua*, Ebro clone 61, Feulgen-stained material.

Figs 49, 50. Vegetative cells in interphase in valve (Fig. 49) and girdle (Fig. 50) view.

Figs 51–53. Three focuses of a gametangium that has dehisced (cf. Fig. 43): four equal nuclei are present, two in each gamete.

Figs 54, 55. Two focuses of a gametangium immediately after meiosis II, showing two binucleate protoplasts lying side-by-side.

Fig. 56. Dehisced gametangium containing rounded gametes (cf. Fig. 28), each with two equal nuclei.

Fig. 57. Young zygote with four equal nuclei.

Fig. 58. Mature zygote (note the more refractile walls compared to Fig. 57), containing only two nuclei.

Fig. 59. Partly expanded auxospore with two unfused haploid nuclei, which have become closely associated at the centre of the auxospore (cf. Fig. 36).

Fig. 60. Fully expanded auxospore before initial valve formation: a single diploid nucleus is present.

Fig. 61. Large cell derived (within a few cell divisions)from an initial cell. The elongate nucleus contains a large central nucleolus.

Young zygotes (Fig. 57) also contained four nuclei, all still apparently equal in size and staining intensity, but older zygotes, distinguishable by their more robust walls (Fig. 58), contained only two nuclei. Expanding auxospores also contained two nuclei, which became paired at the centre (Fig. 59). Only when expansion was more or less complete did the nuclei fuse to restore the diploid condition (Fig. 60). Diploid and haploid nuclei were clearly separable by their size (compare the diploid nuclei of Figs 49, 50, 60 and 61 with the haploid nuclei of Figs 51–59).

For comparison, we examined Geitler's acetocarmine-stained material of '*N. frustulum* var. *perpusilla*' from Illmitz. The stages recorded by Geitler (1970a) were confirmed. Cells in meiotic prophase expanded in girdle view (Fig. 62) and divided after meiosis I. Meiosis II took place in each protoplast to produce four haploid nuclei, which were initially equal in size and staining (Figs 63, 64). Before plasmogamy, however, and in contrast to *N. inconspicua*, one nucleus in each protoplast began to degenerate (Fig. 65), so that young zygotes contained two slightly larger and paler nuclei – the functional gametic nuclei – and two smaller and denser 'pyknotic' nuclei (Fig. 66). Mature zygotes (distinguishable by their thicker walls, as in *N. inconspicua*) contained only two nuclei (Fig. 67), which subsequently became paired but remained unfused, lying on one side at the centre of the auxospore (Fig. 68). The haploid nuclei of '*N. frustulum* var. *perpusilla*' were approximately the same size as the diploid nuclei of *N. inconspicua* (compare Figs 63, 67 and 68 with Figs 49, 50 and 60).

Incunabula, perizonium and initial cell

Acid-cleaned preparations of auxosporulating cells of *N. inconspicua* (clones 61 and 62) showed that the envelopes surrounding the zygotes – the incunabula – contained nonorganic components that resisted oxidation (Fig. 33). These persisted during expansion of the auxospores as two caps, which covered the ends of the auxospore (Figs 34, 69) and sometimes dissociated into numerous curved fragments in our preparations (Fig. 73, arrowheads). Several of Geitler's preparations of '*N. frustulum* var. *perpusilla*' were made by incineration. Those in which the oxidation of organic material

Figs 62–68. '*N. frustulum* var. *perpusilla*': Geitler collection, slides 15/31A (Fig. 67 only) and 15/31B: acetocarmine-stained material from Illmitz.

Fig. 62. Gametangium at diakinesis.

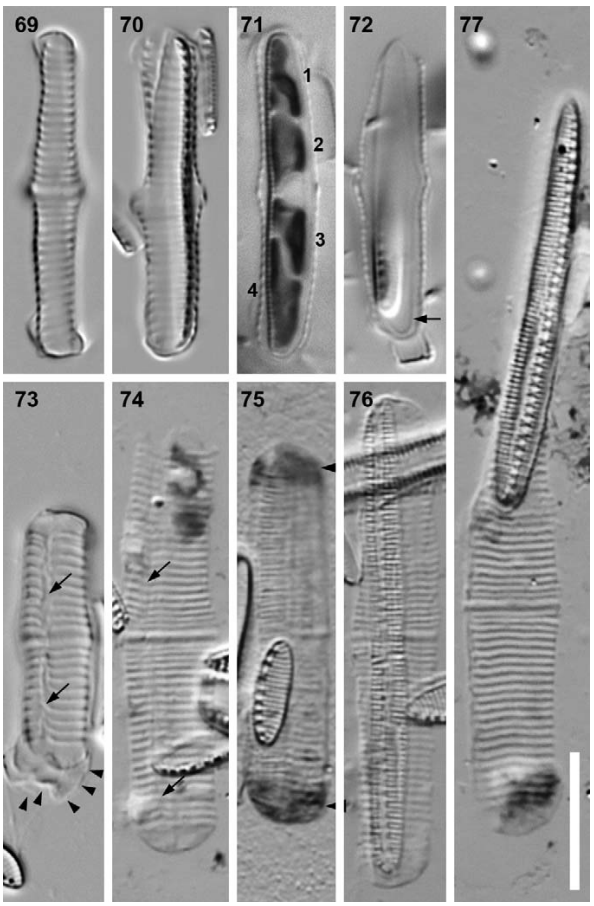
Figs 63, 64. Two focuses of a divided gametangium immediately after meiosis II, showing the four haploid nuclei (two in each protoplast).

Fig. 65. Gametangium with gametes that have begun to round up, each containing a larger functional nucleus (arrow) and a smaller pyknotic nucleus (arrow heads) (cf. Geitler 1970a, fig. 3c).

Fig. 66. Young zygote, containing two larger, functional nuclei and two slightly smaller and denser pyknotic nuclei (arrows) (cf. Geitler 1970a, fig. 3f).

Fig. 67. Mature zygote with rounded wall and containing two closely associated nuclei.

Fig. 68. Young auxospore with two paired haploid nuclei at the centre (cf. Fig. 36).



Figs 69–77. Expanded auxospores and initial cells, including acid-cleaned (Figs 69, 70, 73), incinerated (Figs 74–77) and living (Figs 71 72) material of *N. inconspicua* Ebro clones 61 and 62, and Geitler's Illmitz population of '*N. frustulum* var. *perpusilla*'. Scale bar = 10 μm.

Fig. 69. Clone 62, fully expanded auxospore: note the caps covering the ends of the cell.

Fig. 70. Clone 62, fully expanded auxospore containing initial epivalve (in optical section at right).

Fig. 71. Clone 62, initial cell in girdle view within the perizonium. Four chloroplasts are present (numbered 1–4), arranged in a row (contrast Figs 38–40).

Fig. 72. Clone 62, initial cell escaping from the auxospore, leaving behind a 'husk' within the transverse perizonium (arrow: cf. Geitler 1970a, fig. 3j).

Fig. 73. Clone 61: surface focus of empty perizonium. Note the wide primary transverse perizonial band and very distinct suture (arrow) and the dissociation of the lower cap into numerous curved fragments (arrowheads).

Figs 74–77. Illmitz material on Geitler collection slides 15/28A and 15/28B (Fig. 75 only).

Fig. 74. Perizonium of nearly fully expanded auxospore. Note the narrow transverse perizonial bands in this and Figs 75–77, relative to clones 61 or 62 (Figs 69 and 73) and the poor development of the suture (manifest as a slight inflexion left of centre: arrows).

Fig. 75. Incompletely burnt auxospore showing charred material in the auxospore caps (arrowheads); one of the gametangial valves is visible on top of the perizonium.

was most complete nevertheless preserved the zygote walls and caps as apparently coherent structures (Fig. 74), suggesting that here too the incunabula were silicified. In slide 15/28B of the Geitler collection, the incineration had been less effective and the caps appeared dark (Fig. 75), presumably reflecting charring of organic components of the incunabula.

SEM of the Ebro clones revealed that the incunabula surrounding the young (Figs 78, 79) and mature (Fig. 80) zygotes contained numerous very delicate, more or less circular scales, which separated into two groups during auxospore expansion, forming the caps visible in LM. The incunabula did not contain silicified strips, but fibrous structures were often seen at the open ends of at least one of the gametangial thecae (Fig. 80) and seemed to be continuous with the series of girdle bands.

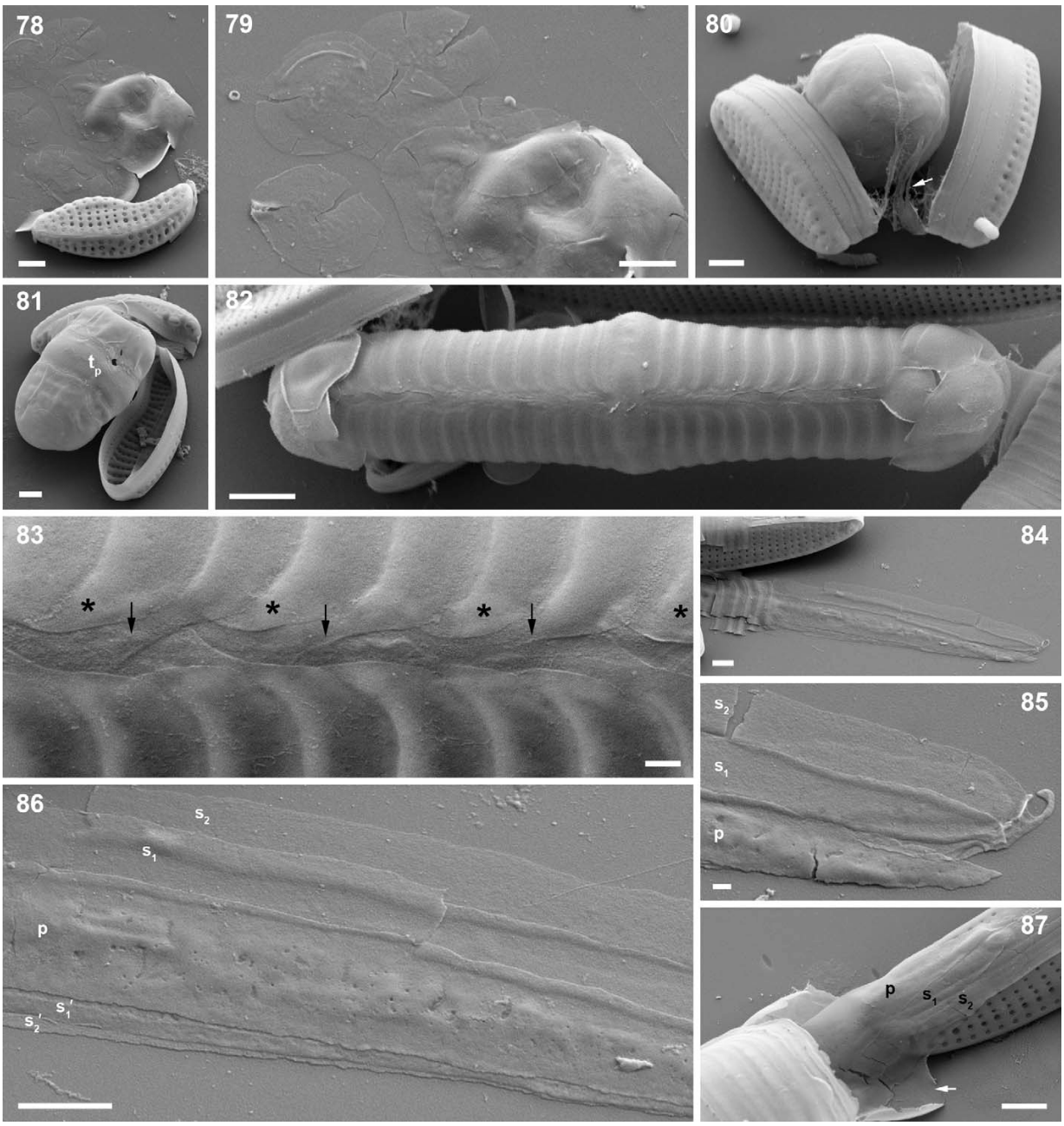
The auxospores of *N. inconspicua* and '*N. frustulum* var. *perpusilla*' possessed a transverse perizonium consisting of a wider primary band and numerous secondary bands (15 or more on each side of the primary band in fully expanded auxospores of *N. inconspicua*, higher numbers in '*N. frustulum* var. *perpusilla*'; Figs 69, 71 and 73 and Figs 74 and 77, respectively). Although the 'var. *perpusilla*' auxospores were longer and generally wider than in *N. inconspicua*, their perizonia were more finely structured, the secondary perizonial bands close to the primary band being particularly narrow (Figs 74, 77; compare Figs 69, 73). In addition, whereas a suture was clearly visible in *N. inconspicua*, lying along one side of the auxospore in suitably orientated specimens (Fig. 73), in 'var. *perpusilla*' the only indication of a suture was a line of slight inflections of the secondary bands (Fig. 74, arrows), accompanied by faint parallel lines that might reflect the presence of a longitudinal perizonium (see below).

Examination of *N. inconspicua* clones 61 and 62 with SEM showed that the primary transverse perizonial band was a complete hoop but that the secondary bands were split rings (Figs 81–83). Both the primary and the secondary bands were plain (or had at most a slight pitting internally, not shown) and lacked fimbriae on their margins (Figs 82, 83). The ends of the secondary bands were curved towards the centre of the auxospore (Fig. 82) and were alternately simple (with tapering curved ends) and more complex (with ends that extended across the suture to finish beneath the ends of the bands on the opposite side of the suture; Fig. 83).

Beneath the suture of the transverse perizonium there was a delicate longitudinal perizonium (Figs 84–87, Supplementary Fig. 2A–C), in which the bands tapered in width towards each pole (Figs 84, 85). The central, primary band was wider than the others and more or less bilaterally symmetrical (Fig. 86, Supplementary Fig. 2B) and bore irregularly positioned pores and fainter slits. Flanking the primary band on one side were two secondary bands (s_1 and s_2), about half the width of the primary band, which were plain or bore irregular slits (Figs 84–87). On the opposite side there were another two, much

Fig. 76. Incompletely silicified epivalve within fully perizonium. Note that, as in clone 62 (Figs 41, 42), the valve does not fill the perizonium as a result of a contraction of the cell before valve formation.

Fig. 77. Initial cell escaping from perizonium.



Figs 78–87. *N. inconspicua*, Ebro clone 61, incunabula and perizonium, SEM. Scale bars = 1 μm, except Figs 82 (2 μm), 83 and 85 (200 nm).
Fig. 78. Gametangial valve and collapsed scale case (incunabula) of zygote.
Fig. 79. Detail of Fig. 78, showing almost plain bands with very slight concentric structuring.
Fig. 80. Mature zygote (with complete scale case) flanked by gametangial thecae (cf. Figs 32, 47); note the fibrous material at the open ends of the thecae (arrow).
Fig. 81. Early stage in auxospore expansion (cf. Fig. 35), showing the wide primary transverse perizonial band (t_p) flanked by three secondary bands on either side. The ends of the auxospore are still covered by incunabular scales.
Fig. 82. Fully expanded auxospore, showing the transverse perizonium, suture (cf. Fig. 73) and incunabular caps.
Fig. 83. Detail of suture, showing the alternation between transverse perizonial bands with simpler curved ends (asterisks) and those with extensions that cross over to end beneath the other side of the suture (arrows).
Figs 84–86. Part of longitudinal perizonium seen from its exterior (Fig. 84) and two details (Figs 85, 86), showing a wide primary band (p) flanked by two wide secondary bands on one side (s_1 and s_2) and two narrow secondary bands on the other (s_1' and s_2'). Note the irregular perforations of the primary band (Fig. 86).
Fig. 87. Detail of initial cell escaping from the perizonium (cf. Fig. 77); labelling as above (for the context of this photograph, see Supplementary Fig. 2D).

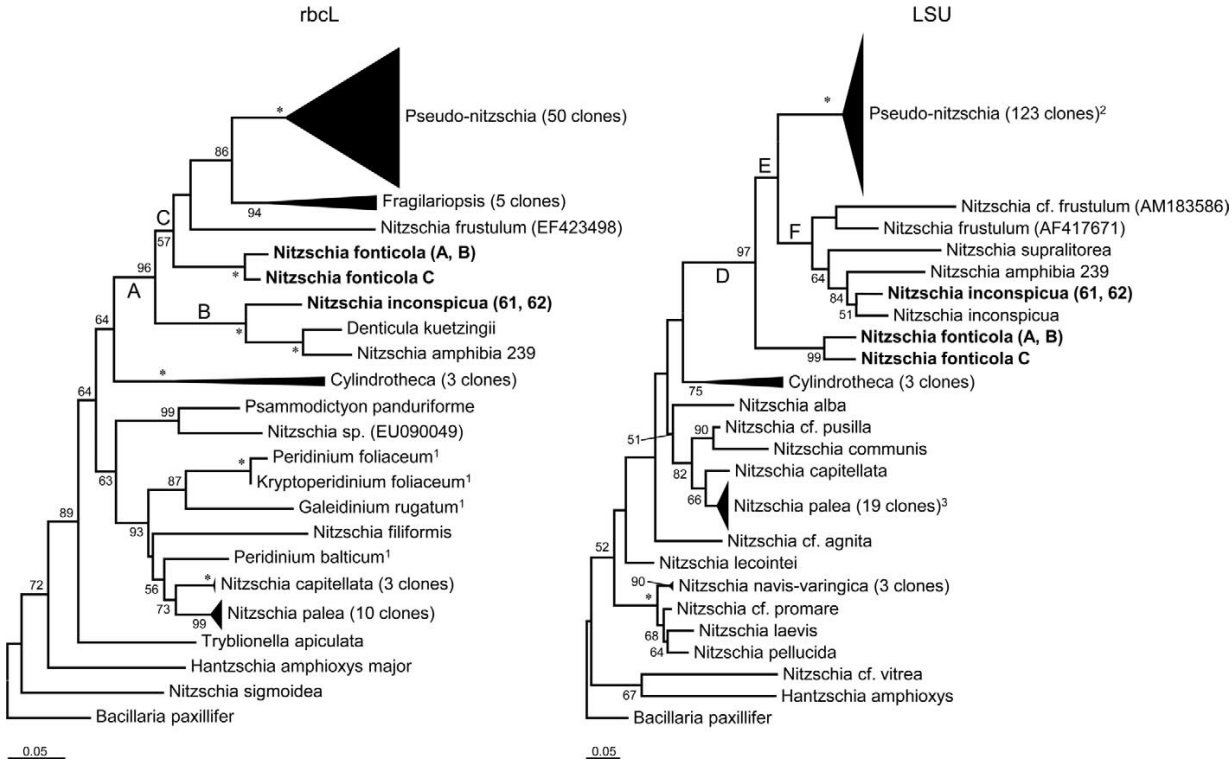


Fig. 88. Molecular phylogeny of *Nitzschia* and related genera inferred from maximum likelihood (RAxML) analysis of *rbc*L and LSU. Paedogamous clones are indicated in bold. Numbers above the branches are RAxML bootstrap proportions when $P \geq 50\%$; * indicates 100%. Branch lengths are proportional to the number of substitutions per site (see scale bar). Some clades were collapsed into triangles for simplification. [1]Endosymbiont of dinoflagellate. [2]Height was reduced to half. [3]'*N. cf. pusilla*' GU732414 is embedded in this clade of 18 clones of *N. palea*.

narrower bands (s_1' and s_2' in Fig. 86; Supplementary Fig. 2C), making a total of five longitudinal bands in all. The longitudinal perizonium is therefore asymmetrical.

After auxospore expansion was complete in *N. inconspicua*, the four chloroplasts became rearranged within the auxospore to lie in a straight line, with a wider separation of the central pair to accommodate the nucleus (Fig. 71). During the formation of the initial valves, the protoplast contracted away from the perizonium, especially at the centre and poles (Figs 41, 42), so that the initial cell was at first a highly elongate ellipsoid, whereas the auxospore was cylindrical, with a slightly expanded centre (Figs 40, 82). The initial valves had arched valve faces in *N. inconspicua* and '*N. frustulum* var. *perpusilla*', with no clear differentiation into valve face and mantle (Supplementary Fig. 2E). Once the initial cell of either taxon had a complete frustule, it usually escaped from the auxospore (Figs 77, 87, Supplementary Fig. 2D), though sometimes it divided once or more *in situ*. Within the vacated perizonium, a 'husk' could sometimes be seen (Fig. 72), echoing the shape of the initial cell that had left. The nature of the husk was not determined, but it seems likely that it comprises more than the longitudinal perizonium or is an entirely separate structure, judging by the shape of the longitudinal perizonial bands shown in Fig. 87 and the fact that no evidence of the structure was found in acid-cleaned or incinerated material (suggesting that it is organic). Similar structures have been illustrated in *Seminavis* (Chepurnov *et al.* 2002, fig. 9a).

The initial cells were much longer in '*N. frustulum* var. *perpusilla*' than in either clone 61 or clone 62 of *N. inconspicua* (Table 1 and compare Figs 7, 76 and 77 with Figs 20, 70 and 71). There was no significant correlation between the lengths of the gametangia and the lengths of the initial cells derived from them in '*N. frustulum* var. *perpusilla*' ($n = 40$, $r = 0.24$, $P = 0.13$). In *N. inconspicua*, some initial cells were strongly bent and/or abnormally structured (9 out of 40 in clone 61, 8 out of 42 in clone 62) and were scored as such before statistical analysis was made of the relationship between gametangial length and initial cell length. When deformed auxospores were included in the analysis, there was a statistically significant relationship between the lengths of the gametangia and initial cells in clone 62 ($n = 50$, $r = 0.32$, $P = 0.03$: smaller gametangia appeared to produce smaller initial cells) but not in clone 61 ($n = 50$, $r = 0.08$, $P = 0.56$). When the deformed auxospores of clone 62 were omitted, however, there was no significant relationship between the sizes of the initial cells and the gametangia ($n = 42$, $r = 0.00$, $P = 0.99$). It seemed that very small gametangia were more likely to produce deformed auxospores than larger ones. Initial cells of '*N. frustulum* var. *perpusilla*' had very slightly but significantly higher mean

striation densities than the two clones of *N. inconspicua* ($P < 0.001$, Tukey *post hoc* test), which did not differ between themselves; however, the range in the Illmitz material completely overlapped those of the *N. inconspicua* clones.

Molecular phylogeny

In *rbc*L and LSU phylogenies (Fig. 88), the genus *Nitzschia* was paraphyletic with respect to several other genera (*Cylindrotheca*, *Denticula*, *Fragilariopsis*, *Psammodictyon*, *Pseudo-nitzschia* and *Tryblionella*), most of which have at one time or another been classified in Nitzschia. The paedogamous clones of *N. inconspicua* appeared within a well-supported clade (96% bootstrap value in rbcL, 97% in LSU, labelled A and D, respectively, in Fig. 88), which was sister to *Cylindrotheca* in both trees.

In the *rbc*L tree, the *N. inconspicua* clones 61 and 62 formed a robust clade (B) with *Denticula kuetzingii* Grunow and *Nitzschia amphibia* Grunow. The other paedogamous *Nitzschia*, *N. fonticola* (studied by Trobajo *et al.* 2006), comprising two genotypes, were contained in a separate clade C, whose support was weak (57%) and which also contained a sequence of '*N. frustulum*', *Fragilariopsis* spp. and *Pseudo-nitzschia* spp.

In the LSU tree, a paedogamous clade of *N. fonticola* diverged first within the clade D, and the rest of clones formed a weak clade E, which further bifurcated into a clade of *Pseudo-nitzschia* and a clade comprising various species of Nitzschia sect. *Lanceolatae*. The paedogamous *N. inconspicua* clones 61 and 62 were situated at the most diverged position in clade F, being sister to another sequence of *N. inconspicua* (from Trobajo *et al.* 2006) within a moderately supported clade (84%) also containing *N. amphibia*.

In order to test whether paedogamy evolved once or at least twice (in the ancestors of *N. fonticola* and *N. inconspicua*, respectively), we ran ML analyses in which the paedogamous clones (*N. fonticola* clones A, B and C and *N. inconspicua* clones 61 and 62) were constrained to be monophyletic. Hypothesis testing by AU and NP tests rejected the constrained trees as significantly worse than the best tree (Supplementary Table 3) and hence rejected a single evolutionary origin of paedogamy in *Nitzschia*. A second test was made of the LSU data set in which the genotype of *N. inconspicua* sequenced by Trobajo *et al.* (2006; GenBank AM182195) was also included in the constrained 'paedogamous clade', together with the Ebro *N. inconspicua* and *N. fonticola*. Again, the constrained topology was rejected as significantly worse than the best tree (Supplementary Table 3).

DISCUSSION

The remaining material of '*N. frustulum* var. *perpusilla*' cannot be used to obtain molecular data to study the relationship between this diatom and *N. inconspicua*, nor is it possible to examine Geitler's material by SEM. In LM, *N. inconspicua* and '*N. frustulum* var. *perpusilla*' are very similar in valve shape and pattern, but they differ in several respects that indicate genetic divergence. The most striking difference in the vegetative cells is the maximum length achieved: the initial cells of '*N. frustulum* var. *perpusilla*' (*c.* 30 μm) are much longer than those produced by clones 61 and 62 of *N. inconspicua* (*c.* 21 μm). The sizes to which auxospores expand and the maximum size of the gametangia are fairly constant within clones of pennate diatoms and considered to be largely under genetic control (Geitler 1932; Edlund & Bixby 2001, p. 185; Chepurnov *et al.* 2004), which allows their use as taxonomic characters. It is true that there is a tendency in some species for smaller gametangia to give rise to smaller initial cells (reviewed by Edlund & Bixby 2001), but there was no correlation between gametangium size and initial cell size in our analyses of the Illmitz material and the Ebro clones, except once in clone 62 when obviously deformed auxospores were included. Furthermore, the Illmitz and Ebro initial cells, differing in length by *c.* 8–9 μm, were derived from gametangia that differed by only *c.* 2 μm (Table 1). Thus, size dependency between gametangia and initial cells cannot account for difference in the lengths of the initial cells between the Illmitz and Ebro material.

Other features also point to a differentiation between the Illmitz material and the Ebro clones. These are that, in the Illmitz material, (1) the superfluous nuclei from meiosis I degenerate earlier, (2) the auxospores are wider, (3) the perizonium is more finely structured and the suture is indistinct, and (4) the mean striation density is very slightly higher, at least in large valves, although the ranges overlap almost completely. It seems also that the nuclei of Illmitz cells are larger than in the Ebro clones (the haploid gamete nuclei of Illmitz cells being approximately the same size as the vegetative diploid nuclei of the Ebro clones), which could suggest that the Illmitz population is tetraploid (evidence in angiosperms indicates that nuclear volume is correlated with genome size; Jovtchev *et al.* 2006), and this would be consistent also with the much larger size of the Illmitz initial cells (cf. Connolly *et al.* 2008). However, the preparations were not made using the same fixation and staining protocol and are much older, and this could have differentially affected nuclear size, although there was no evidence of swelling of the cells themselves. Within each clone or population, characteristics (1)–(4) appeared constant, and these features also seem to show little plasticity in other diatoms. For example, the superfluous haploid nuclei are always persistent in some *Navicula* species (Poulíčková & Mann 2006); initial valves often have characteristic shapes, which may differ from those of the vegetative cells that are derived from them (e.g. Mann 1989); and striation density is relatively unaffected by environmental conditions, including salinity (Trobajo *et al.* 2004, 2011), which differed between the Ebro and Illmitz cultures.

We conclude, therefore, that there are genetic differences with respect to maximum size, gametogenesis, auxospore structure, and perhaps also mean stria density, between *N. inconspicua* and '*N. frustulum* var. *perpusilla*'. Whether this variation can be treated as intraspecific (see also discussion in Supplementary Text) or reflects a more distant relationship (*N. frustulum* and *N. inconspicua* are clearly separated in the *rbc*L and LSU trees despite their morphological similarity, clones 61 and 62 being less closely allied to *N. frustulum* than to the morphologically dissimilar *N. amphibia*: Fig. 88) cannot be determined without further sampling, preferably including material from Lake Neusiedl.

Table 2. Comparisons of development and auxospore structure among paedogamous *Nitzschia* populations.

	N. inconspicua Ebro clones	'*N. frustulum* var. *perpusilla*' Illmitz material	*N. fonticola*[1]
Timing of degeneration of superfluous haploid nuclei	after plasmogamy, in zygote	before plasmogamy, in gametes	before plasmogamy, in gametes
Zygote shape	spherical	spherical	ellipsoidal
Organic incunabula	thin, not obvious	thin, not obvious	thick
Siliceous incunabula	round scales	present but details unknown, not strips	ball of strips
Persistence of siliceous incunabula during auxospore expansion	as polar caps	as polar caps	dispersed along auxospore
Distal margin of transverse perizonial bands	plain	?	fimbriate
Frequency of irregularly curved auxospores and initial cells	low	low	high
Shape of ends of transverse perizonial bands	strongly curved (suture obvious in LM)	scarcely curved (therefore suture indistinct)	strongly curved (not observed in LM because of incunabula)

[1] Source of information: Trobajo *et al.* (2006).

The nature, consequences and evolution of paedogamy in *Nitzschia*

Our data indicate that the Ebro clones of *N. inconspicua* are strictly paedogamous because no pairing was observed at any time, either in monoclonal cultures or in mixtures of different clones. There have been two previous reports of paedogamy in *Nitzschia*: (1) Geitler's (1970a) paper on the Illmitz material of '*N. frustulum* var. *perpusilla*', re-examined here, and (2) Trobajo *et al.*'s (2006) study of three clones of *N. fonticola*. The main features of the process are by definition similar throughout: a single gametangium produces two gametes, which fuse with each other to produce a single zygote and hence a single auxospore. Moreover, there are also some agreements in detail between the different reports of paedogamy in *Nitzschia*. For example, in all three taxa, the gametangia elongate along the pervalvar axis – that is, they become wider in girdle view – as they enter meiosis (Fig. 27; Geitler 1970a, p. 124; Trobajo *et al.* 2006, cf. fig. 5c with fig. 4b). Furthermore, the 'cruciate' arrangement of the four chloroplasts during auxospore expansion in *N. inconspicua* (Figs 35–39) is also evident in living '*N. frustulum* var. *perpusilla*' (Geitler 1970a, fig. 2f, g), and the chloroplasts of mature auxospores lie in a single line (Fig. 71; Geitler 1970a, fig. 2h, i). We noted too that one of the gametes in the Ebro clones often had a more 'plastic' morphology than the other, deforming its shape or producing pseudopodium-like extensions while its sibling remained unaltered, and it appears that this may also occur in '*N. frustulum* var. *perpusilla'*, judging by Geitler's illustrations (Geitler 1970a, figs 2b, c).

However, there are also differences between the three paedogamous *Nitzschia*, which are evident in the morphology of the auxospores and their envelopes (incunabula and perizonium) and in the timing of the degeneration of the two superfluous haploid nuclei (Table 2). The differences in the incunabula are particularly striking: *N. fonticola* has large numbers of silica strips around its zygotes, which resemble balls of wool (Trobajo *et al.* 2006, fig. 8b, c), whereas the Ebro strains of *N. inconspicua* and probably also '*N. frustulum* var. *perpusilla*' have incunabula containing quite small numbers of simple round scales.

Less obvious than the incunabular differences but potentially very significant is the contrast in the timing of nuclear degeneration, with all four nuclei persisting into the zygotes in *N. inconspicua* but only two remaining uncontracted and apparently functional in '*N. frustulum* var. *perpusilla*' (Figs 65, 66; Geitler 1970a, fig. 3c–f) and *N. fonticola* (Trobajo *et al.* 2006, fig. 5e and p. 1362). We will refer to the kind of paedogamy in which all four nuclei survive into the zygote as 'immature' because the gametes retain an earlier stage of development at plasmogamy relative to those in which one haploid nucleus has already degenerated, which we will call 'mature' paedogamy. The genetic consequences of this difference may be considerable. If the four nuclei are indeed all functional after plasmogamy, as they appear to be in the immature paedogamy of *N. inconspicua*, then haploid nuclei of the tetrad can presumably pair in any combination to produce new diploid progeny, and successive generations will therefore progress rapidly towards complete homozygosity, regardless of the positions of chiasmata. Any advantages of heterozygosity will therefore be lost, and deleterious recessive mutations will be exposed to selection. However, in mature paedogamy, where one nucleus degenerates in each gamete before they fuse, intratetrad fusion of nuclei in the auxospore is constrained to be between haplotypes that have segregated at the first division of meiosis. Consequently, for any genes completely linked to the centromeres (through simple proximity or suppression of crossing over), any existing heterozygosity can be maintained, and recessive deleterious mutations can be masked. Hood & Antonovics (2004) suggest [from studies of the smut fungus *Microbotryum violaceum* (Persoon) Deml & Oberwald and theoretical considerations] that any automictic process arising to enforce intratetrad fusion between genotypes segregating at meiosis I (as in the mature paedogamy of the Illmitz population and *N. fonticola*) is likely to become obligate. This is because, as deleterious alleles accumulate at the centromeres, there is 'an increasing fitness cost of mating between tetrads or to closely related diploid genotypes [as can occur in homothallic diatoms] because exposing the [deleterious] alleles to homozygosity would occur at a very high rate. Thus, a feedback process may quickly develop, with mating within the tetrad becoming more frequent, the extensive accumulation of deleterious recessive

mutations, and the system being driven ever further from the ability to produce viable progeny by mating outside the tetrad' (Hood & Antonovics 2004). On this basis, it is unlikely that the Illmitz population and Trobajo *et al*.'s (2006) populations of *N. fonticola*, both with mature paedogamy, would have ever exhibited allogamous reproduction.

The presence of paedogamy in two lineages of *N. fonticola* and in two clones of *N. inconspicua* prompted us to use our molecular data not only to document the identity of the clones used but also to examine whether paedogamy had a single origin in *Nitzschia*. The two gene trees indicate that *N. fonticola* and *N. inconspicua* belong to the same major clade of Bacillariaceae and are more closely related to each other than they are to some other, superficially similar species classified in *Nitzschia* sect. *Lanceolatae*, such as *N. capitellata* Hustedt in Schmidt *et al*., *N. palea* (Kützing) W. Smith and *N. communis* Rabenhorst. However, *N. fonticola* and the Ebro *N. inconspicua* clones are not sister species, and the clade containing them also contains genera and species that seem to be or contain fully sexual species. Thus, in this clade, heterothallic or homothallic reproduction has been reported to be characteristic of several *Pseudo-nitzschia* species (e.g. Davidovich & Bates 1998; Amato *et al*. 2005; Chepurnov *et al*. 2005; Quijano-Scheggia *et al*. 2009), and allogamy (without information about whether this is hetero- or homothallic) is known in *Nitzschia amphibia* (Geitler 1969; D.G. Mann, unpublished observations of UK material) and *D. tenuis* (Geitler 1953). Allogamy is also likely in *Fragilariopsis kerguelensis* (O'Meara) Hustedt, though the early stages of auxosporulation have not been observed (Assmy *et al*. 2006). *Cylindrotheca*, which is the sister group to the clades containing the paedogamous *Nitzschia* species (clade A of the *rbc*L tree, clade D of the LSU tree), has also recently been shown to contain allogamous species (Vanormelingen *et al*. 2013).

Unfortunately, information on auxosporulation is not available for all the species included in our phylogenetic trees; for example, as far as we know, there are no auxosporulation data for *N. supralitorea* Lange-Bertalot or the '*N. frustulum*' clones within clade D of the LSU tree. This lack of information prevents direct estimation of ancestral character states. However, we think that it is reasonable to make the assumption that the change from allogamy to paedogamy is essentially irreversible once it is established in a population for the following reasons. Allogamy is a complex process, involving attraction and recognition between sexualized cells; these are not required in a paedogamous diatom and do not occur in *N. inconspicua*, '*N. frustulum* var. *perpusilla*' or the clones of *N. fonticola* studied by Trobajo *et al*. (2006). There must also be mechanisms in allogamous diatoms to facilitate and control fertilization. For example, *N. amphibia* cells form copulation tubes, and *D. tenuis* forms a capsule around the paired cells, creating an environment that allows the gametes to find and fertilize each other. In addition, diatoms that produce two gametes per gametangium (e.g. *N. flexoides*, *N. sigmoidea*, *N. recta*, *N. palea*, *Pseudo-nitzschia* species; Geitler 1968; Mann 1986; Amato *et al*. 2005; Trobajo *et al*. 2009) must possess mechanisms that prevent fusion between gametes from the same gametangium. Again, the paedogamous *Nitzschia* exhibit none of these features: no pairing was ever seen in clones 61 or 62, the Illmitz material or *N. fonticola* (Trobajo *et al*. 2006); special structures to facilitate plasmogamy are not produced; and the unknown mechanism that prevents selfing between gametes within the gametangia of allogamous diatoms is by definition absent in any paedogamous form. Hence, evolution of allogamy from paedogamy would require the acquisition of many new mechanisms and processes, whereas the reverse principally entails loss.

Assuming, therefore, that paedogamous populations evolve from allogamous ones but not vice versa, we can say that any clade containing even one allogamous sexual diatom must have arisen from an allogamous sexual ancestor. On this basis, the ancestor of *rbc*L clades B and C must have been allogamous (B contains the allogamous *N. amphibia*, C contains allogamous *Pseudo-nitzschia*), and so must LSU clades D, E and F. Therefore, in order to use the two molecular data sets to test whether paedogamy is likely to have evolved once or at least twice in *Nitzschia*, it is sufficient to test whether trees constrained to make {*N. fonticola* + *N. inconspicua*} monophyletic are significantly less likely than the best trees presented in Fig. 88. Because they are indeed significantly less likely (Supplementary Table 3), whether or not the second *N. inconspicua* genotype (for which we have no information about auxosporulation) is included in the constrained clade, we conclude that paedogamy has probably evolved at least twice in *Nitzschia*.

There is a further piece of evidence that is consistent with this conclusion because it indicates that the paedogamous *N. fonticola* populations may indeed be closely allied to an allogamous form, not to other paedogamous ones. This is the report by Geitler (1932, pp. 168–172) of allogamy in a population from the Seebach at Lunz, Austria, which he identified as *N. fonticola*. Species definitions in *Nitzschia* have changed considerably since 1932, and there can be no certainty that Geitler's allogamous population and the paedogamous populations (from three sites in Spain and the United Kingdom) are closely related. However, all of them seem to have strip incunabula (Trobajo *et al*. 2006), which are absent in the other members of *rbc*L clade A and LSU clade D, where auxospores have been investigated (*N. amphibia*: Geitler 1969; *Pseudo-nitzschia*: Kaczmarska *et al*. 2000; Amato *et al*. 2005; *Fragilariopsis*: Assmy *et al*. 2006), and also in *Cylindrotheca* (Vanormelingen *et al*. 2013), which is the sister group to clades A and D.

Besides the three paedogamous *Nitzschia* discussed above, there are a few other reports of uniparental auxosporulation (either automixis or apomixis) in *Nitzschia* sect. *Lanceolatae*: Geitler 1932 (p. 172) observed it in a marine species from Naples, and Geitler (1932, p. 220) also mentioned a previous record by Miquel, which we have not examined. Furthermore, Wiedling (1948) noted that clones of several sect. *Lanceolatae* species did not decline in size in culture, so that (if this behaviour is representative of what happens in nature) they may be wholly asexual. Overall, then, the number of species of *Lanceolatae* reported that appear to have reduced or no sexuality is roughly equal to the number known to show biparental auxosporulation (there are roughly seven each, though counting is problematic because of taxonomic uncertainties; data from Geitler 1932, 1973; Wiedling 1943, 1948; Trobajo *et al*. 2006, 2009; this article). This contrasts with the Cymbellales (sensu Round *et al*. 1990), which are relatively well studied with respect to the life cycle and in

which most species so far (of *Cymbella* sensu lato, *Gomphonema*, *Didymosphenia*, *Placoneis*, *Anomoeoneis* and *Rhoicosphenia*) have proved to be allogamous (summaries by Geitler 1932, 1973; also Mann & Stickle 1995). A high proportion of uniparental reproduction in *Nitzschia* sect. *Lanceolatae* could help explain the well-known taxonomic difficulty of the group, as suggested previously by Trobajo *et al*. (2006), each clade of uniparentally reproducing *Lanceolatae* comprising an array of lineages with small fixed differences in morphological characteristics.

Incunabula and perizonium structure in raphid diatoms

Scaly incunabula have been reported from the auxospores of a few raphid diatoms, including *Sellaphora* (Mann *et al*. 2011), some *Pinnularia* (Ishii *et al*. 2012), *Diploneis* (M. Idei, personal communication), *Pseudo-nitzschia multiseries* (Hasle) Hasle (Kaczmarska *et al*. 2000), *Nitzschia longissima* (Brébisson in Kützing) Grunow (Kaczmarska *et al*. 2007) and now *N. inconspicua*. The scales sometimes bear radiating systems of branching ribs, for example, in *Diploneis* and *Sellaphora*, but in *N. inconspicua* and *N. longissima* they are almost plain or irregularly porous. The phylogenetic diversity of the genera listed above (for a recent three-gene phylogeny, see Theriot *et al*. 2010) suggests that the potential of the zygote to form scales may be a symplesiomorphy in raphid diatoms. Furthermore, more elaborate silica structures that were probably derived from scales and comprise helmet- and plate-like structures are present in the incunabula of *Neidium* (Mann & Poulíčková 2009) and almost certainly also in *Biremis* (Mann 1993), *Muelleria* and *Scoliopleura* (Edlund & Spaulding 2006).

However, even closely related species can differ in incunabular structure. For example, *Pseudo-nitzschia delicatissima* (Cleve) Heiden in Heiden & Kolbe seems to have purely organic incunabula (Amato *et al*. 2005), and *Nitzschia fonticola* possesses a tangle of silica strips rather than tightly imbricating scales. The diversity of incunabula already known, from scales and strips to thin organic coverings (e.g. in *Navicula*; Poulíčková & Mann 2006), and the variation that occurs between closely related diatoms are intriguing and suggest that the incunabula are under particularly active selection. The functions of the incunabula are unknown but presumably include protection of the zygote while it undergoes internal reorganization before expansion and possibly the creation of nonspherical shapes in the zygote (Poulíčková *et al*. 2007). The incunabula may also prevent fusion of the zygote with further gametes (avoiding polyspermy). Hence, in seeking the explanation for incunabular diversity, it may be profitable to look for correlations between incunabular form and other characteristics of sexual reproduction, such as whether the gametes and zygotes are protected within mucilage capsules or whether the maturing zygote develops anisometrically (see also Poulíčková *et al*. 2007). For this to be possible, however, wider sampling of raphid taxa is necessary.

Standing in sharp contrast to the variability of the incunabula is the monotony of perizonium structure in pennate diatoms. Where studied in sufficient detail, the transverse perizonium almost always consists of a primary band flanked on both sides by many narrower secondary bands. The principal exception is in the Surirellales, where the transverse series develops unilaterally from a single polar cap (Mann 1987; Watanabe *et al*. 2012). Similarly, the longitudinal perizonium is also rather uniform, usually consisting of a wide primary band flanked symmetrically on either side by two secondary bands. The transverse perizonium of *N. inconspicua* is like that of other pennate diatoms studied so far (von Stosch 1962; Mann 1982; Mann *et al*. 2011) except in one respect, namely the alternation of two types of ends (each band having unequal ends), which overlap to create a complete and intricate covering to the suture that as yet has no parallel in other diatoms. It is possible that similar complexity has been missed elsewhere because of a lack of resolution in SEM (Mann *et al*. 2011), but at least in *Neidium* (Mann & Poulíčková 2009) it seems that no alternation is present.

The longitudinal perizonium of *N. inconspicua* is more unusual, being asymmetrical, with two wide secondary bands on one side of the primary band and two narrow ones opposite. Nothing similar has been reported before. Trobajo *et al*. (2006) reported the existence of a longitudinal perizonium in *N. fonticola* but did not document the structure and arrangement of the bands, and the structure of the longitudinal perizonium is similarly unclear in *N. longissima* (Kaczmarska *et al*. 2007). Perhaps the asymmetry of the longitudinal perizonium in *N. inconspicua* arises through the same kinds of developmental processes as occur in valve morphogenesis in *Nitzschia*, where the pattern centre (the raphe-sternum) becomes displaced during valve formation to lie near one margin (see Pickett-Heaps 1983; Mann 1984) rather than remaining central as in most raphid diatoms. It will be interesting to see whether asymmetrical longitudinal perizonia are present in other diatoms with strongly dorsiventral valves.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found online at http://dx.doi.org/10.2216/12-077.1.s1.

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