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# The oldest raphe-bearing diatoms: Evidence from the Upper Cretaceous of western and northern Canada

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ABSTRACT

Diatoms, with their unique siliceous cell walls, are among the most successful eukaryotic lineages on Earth. They inhabit virtually every wet environment and are central organisms in aquatic food webs and biogeochemical cycles. A highly diverse number of species with an impressive array of morphological designs have evolved since the late Mesozoic, and especially since the Late Cretaceous. Pennate diatoms comprise the largest clade, and include those forms that evolved a raphe, an advancement that yielded motility and the ability to move across surfaces. We report multiple raphe-bearing freshwater diatoms from two Cretaceous fossil localities in Canada, the Battle Formation in Alberta (67Ma), and the Wombat locality in the Northwest Territories (82 Ma). A new species of the genus *Adlafia*, *A. wolfeii* sp. nov., is reported from the Battle Formation. Multiple species of the genus *Eunotia* were uncovered from the Wombat locality pushing back the minimum earliest age for this taxon by ~30 Ma. The discoveries represent two of the oldest known records of raphe-bearing diatoms and support the hypothesis that the raphe evolved in a freshwater environment during the Cretaceous. These new records are placed in context with previous fossil records, and provide new age constraints for use in linking diatom molecular-based phylogenies to the geological record.

## 1. Introduction

Diatoms are unicellular, photosynthetic protists that have evolved into one of the most successful eukaryotic lineages on Earth. Distinguished by their unique siliceous cell wall, diatoms inhabit virtually every wet or moist environment, both freshwater and marine, including ponds, lakes, streams, rivers, bogs, marshes, soils, as well as the full array of marine habitats. As primary producers, they can dominate planktic, periphytic, and benthic environments becoming important components of aquatic food webs and biogeochemical cycles, including carbon and silica cycling (Field et al., 1998; Armbrust et al., 2004; Falkowski et al., 2004; Katz et al., 2005). Because the taxonomically-diagnostic siliceous components enhance their presence in the fossil record, diatoms form a cornerstone of paleobiological research and are important bioindicators of environmental change on time scales ranging from hundreds to millions of years (Katz et al., 2005; Smol and Stoermer, 2010).

The precisely sculptured siliceous cell wall is the signature feature of diatoms. The wall consists of two overlapping and closely fitted pieces called valves that are held in place by additional open or ring-like structures, the girdle elements, to form the complete wall referred to as the frustule (Round et al., 1990). Although diverse arrays of shapes have evolved, diatoms are often informally divided into groups based on the symmetry of the valve. The centric diatoms are composed of forms with radial shapes, the bipolar diatoms possess two parts of the valve each with a radial design, and the pennate diatoms have a bilateral symmetry. Pennate diatoms are further divided into araphid and raphid forms, the latter group possessing a structure known as the raphe. The raphe consists of two elongated slits that penetrate the valve wall and are typically arranged in a linear or end to end arrangement. The raphe is used in movement, giving raphid pennates the ability to move over surfaces, a capability not shared by other diatoms.

Questions surrounding the age of the diatoms, as well as when the higher taxonomic groups (e.g. radial and bipolar centrics, araphid pennates and raphid pennates) each evolved, have long intrigued scientists. Incorporating molecular data into phylogenetic studies has greatly enhanced our understanding of diatom

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phylogeny, evolutionary history, and impacts of past climate states. Early studies using molecular data yielded widely divergent estimates for the age of diatoms, ranging from ca. 400 Ma in the middle Paleozoic (Sorhannus, 1997) to 164 Ma in the Middle Jurassic (Kooistra and Medlin, 1996). These early estimates suffered from a small number of taxa and limited molecular data (Alverson and Theriot, 2005). Recent works based on significantly more taxa have largely narrowed the time frame to the early Mesozoic, most likely between the Early Triassic (i.e. 250 Ma) and Early Jurassic (i.e. 183 Ma) (Julius, 2007; Medlin, 2016). A more recent and robust study by Nakov et al. (2018), that incorporated 1151 taxa, 11 gene sequences, and 38-age-calibration points, yielded a crown age for the evolution of the diatoms at the Triassic-Jurassic boundary (190 Ma) and a stem age estimate of 199 Ma, both within the range estimated in the previous works. Still, an analysis by Brown and Sorhannus (2010) predicted a much earlier origin near the Devonian–Carboniferous boundary. The Brown and Sorhannus (2010) study included far fewer diatom taxa compared to the more diatom-focused works (e.g. Medlin, 2015, 2016; Nakov et al., 2018). However, the scope of their study differed in that it focused on the full complement of photosynthetic heterokonts (17 lineages of which the diatoms are one), and included age-calibration points that encompassed many of the lineages. Although numerous questions remain, additional evidence from the fossil record coupled with improved age-calibration markers, and future improvements to molecular techniques, will continue to strengthen the coupling of the diatom phylogeny to geologic time (Williams, 2007; Nakov et al., 2018).

One consistent finding among the molecular studies, is that araphid pennate diatoms developed after the non-pennate forms, and then graded into the younger and monophyletic clade consisting of raphid pennate forms (Alverson and Theriot, 2005; Kooistra et al., 2007; Sorhannus, 2007; Medlin, 2015; Nakov et al., 2018). The monophyletic nature of the raphid diatoms implies that the raphe evolved only once. Further, molecular clock results estimate that araphid pennates likely originated in the Early Cretaceous, and raphid pennates later in the Cretaceous (Sorhannus, 2007; Medlin, 2015). More recently, Nakov et al. (2018) estimated crown (stem) ages for araphid and raphid diatoms of 136 (148) Ma and 110 (129) Ma, respectively. The age estimates for the evolution of different diatom lineages yield hypotheses that can be tested using the fossil record.

Pennate diatoms bearing a raphe belong to the class Bacillariophyceae, by far the largest and most diverse class of diatoms (Round et al., 1990). This class is further divided into two subclasses, the Bacillariophycidae and the Eunotiophycidae, based primarily on differences in the structure of the raphe (Mann, 1984; Round et al., 1990). For taxa belonging to the Bacillariophycidae (naviculoid type), the raphe slits are long and positioned within the sternum, or pattern center, with transapical ribs and striae extending out from the sternum on each side. In most cases, the proximal ends of the pair of raphe slits terminate close to each other within the center nodule of the valve. Over geologic time, numerous variations on the basic raphe system have evolved, including association with ribs, modifications to the ends of the raphe slits, and development of raphe fissures. In contrast, the raphe slits in the Eunotiophycidae are considerably shorter, reduced, and positioned to one side of the sternum, such that they interrupt the ribs and striae that radiate from this side of the sternum. In most cases, e.g. species belonging to the family Eunotiaceae, the short raphe slits are restricted to the apical ends of the valve and situated on the mantle (eunotioid type). In addition to the raphe system not being integrated into the central sternum, taxa in the Eunotiophycidae are unique among raphe-bearing diatoms in that they possess rimoportulae, a primitive structure found in many centric and araphid pennate species (Round et al., 1990; Kociolek, 2000). The basic structure of a rimoportula is a tube through the valve that opens externally as a simple pore, and internally as an expanded slit often on the end of a short stalk.

The objectives of this study are to: 1) summarize the records of raphe-bearing diatoms from the Eocene or older; 2) describe a new raphe-bearing diatom belonging to the genus *Adlafia* Moser, Lange-Bertalot & Metzeltin from the Cretaceous Battle Formation; 3) present new records for the genus *Eunotia* Ehrenberg from the Cretaceous Wombat locality and; 4) discuss the new findings with respect to the evolutionary history of raphe-bearing diatoms.

## 2. Site descriptions

### 2.1. Battle Formation

Details of the Battle Formation are given in Siver et al. (2018) and references therein. Briefly, outcrops of the Battle Formation are found in southern portions of the provinces of Alberta (AB) and Saskatchewan (SK) (Canada). The formation is a massive, dark brown to black claystone deposit, ranging in thickness from 3 to 12 m (Irish, 1970; Gibson, 1977). It is stratigraphically situated immediately above the Maastrichtian Whitemud Formation and just below the Maastrichtian-Paleocene Scollard and Ravenscrag Formations (Binda, 1970; Lerbekmo et al., 1987). The formation is about 50 m below the K/Pg boundary (Binda, 1970). A distinctive silicified tuffaceous layer, the Kneehills Tuff, was deposited in the upper portion of the Battle Formation. K/Ar dating of sanidine and biotite minerals in the Kneehills Tuff was used to estimate the age of the formation to be 66.5 Ma (Allan and Sanderson 1945; Lerbekmo et al., 1987). More recently, high-precision U–Pb CA–ID–TIMS dating of the deposits and age modeling indicate a slightly older age range of 66.88–67.20 Ma (Eberth and Kamo, 2019). The stark contrast between the Battle (dark brown) and Whitemud Formations (white) is striking and makes the boundary between these formations easy to recognize.

Samples for the current study are from a 3 m outcrop of the Battle Formation located north of the town of Gleichen, AB, on Range Road 231, and adjacent to the Hamlet of Gleichen water reservoir (Fig. 1; 50° 52′49.7″ N/113° 04′21″ W). The portion of the Battle outcrop sampled in this study was approximately 500 m from the reservoir, which was machine-dug approximately 20 years prior to sampling. The outcrop was situated approximately 5 m above the level of the reservoir and topped by prairie grasslands. The Whitemud Formation and Kneehills Tuff zone were clearly visible at the site, referred to as “GP.” The outcrop was systematically sampled every 20 cm starting from the top of the Whitemud Formation, with samples sequentially labeled as GP1, GP2, etc through to the top of the Battle Formation at GP14 (Fig. 2A). We dug into the formation at each level using a pick and shovel in order to retrieve fresh rock samples with cleaned surfaces that had not been exposed to the atmosphere. The rocks were dense and intact across the formation.

### 2.2. Wombat locality

Details of the Wombat fossil locality are given in Grande et al. (2022) and are briefly summarized below. The Wombat locality represents the remains of a lacustrine waterbody contained within a kimberlite maar (64.92° N, 110.45° W) situated in the Slave craton within the Lac de Gras kimberlite field in the Northwest Territories, Canada (Fig. 1). The buried Wombat maar deposits examined as part of this study were retrieved from a drilled core, CH93-29, taken in 1993 for diamond exploration of the kimberlite

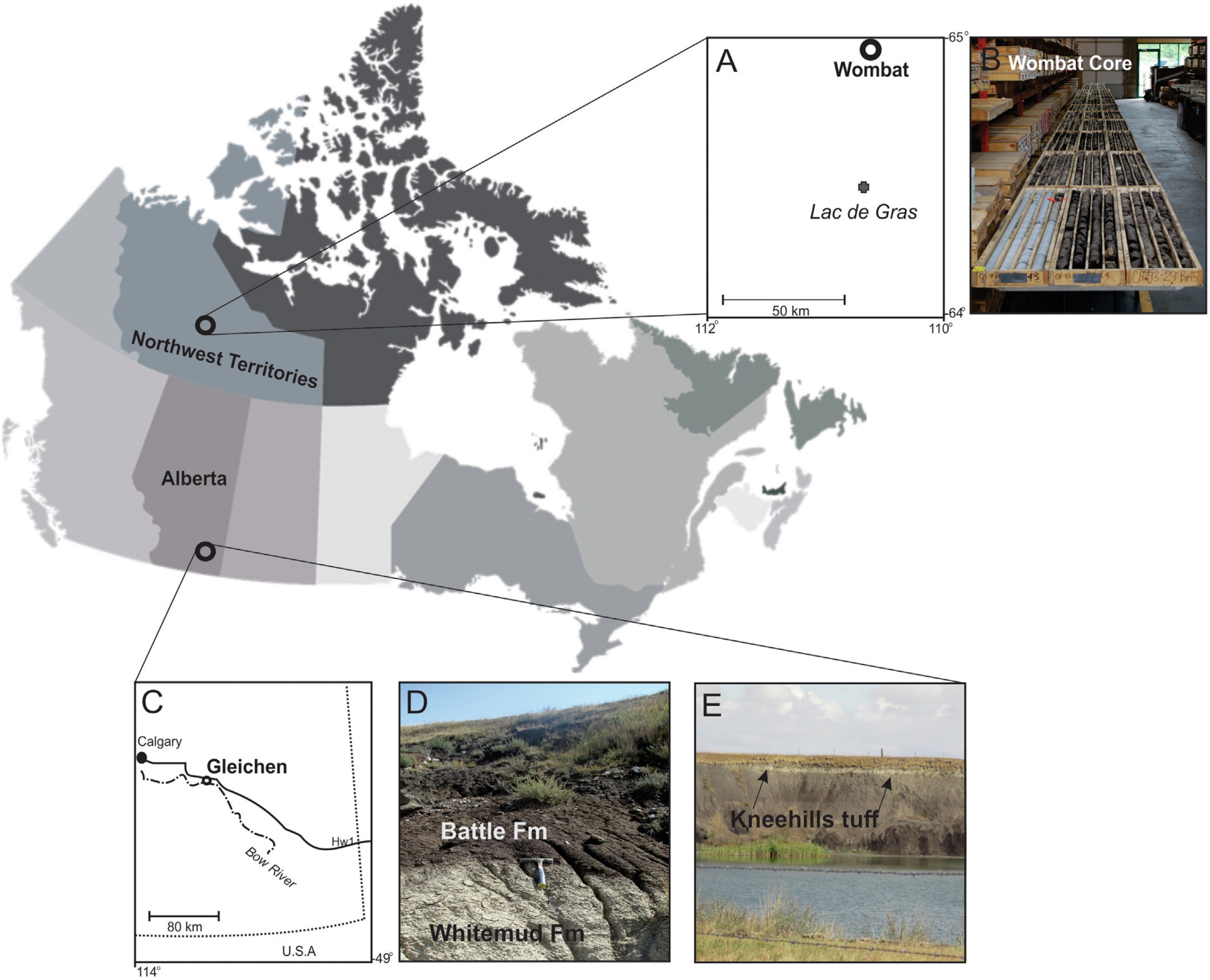


**Fig. 1.** Map depicting the geographic locations of both the Gleichen Pond (GP) outcrop of the Battle Formation, and the Wombat fossil locality. A) Location of the Wombat locality. B) The Wombat core. Note the transition from granite country bedrock to lacustrine mudstones in the core box to the left marked with orange tape. C) Location of the Gleichen Pond (GP) outcrop of the Battle Formation. D) Image depicting the transition between the Whitemud Formation and the Battle Formation. E) Image showing the remains of the Kneehills tuff).

rock (Fig. 2B). Core CH93- 29, archived at Connecticut College (New London, USA), contains ~195 m (vertical equivalent) of crater lake facies stored in core boxes, each with four 1 ½ m length channels. Samples are identified with a three-number code: the first represents the core box number; the second is the channel in the box (with 1 representing the stratigraphically highest channel); and the third represents the depth of the sample within the channel. For example, W62-2-25 represents a sample taken from 25 cm from the top of the core section in channel 2 of box 62. Samples containing remains of *Eunotia* examined in this study came from ten strata, two from box 50 and eight from boxes 62–65 as outlined below. The core samples in box 50 consisted of hard, laminated silty mudstone from about 190 m below ground level. The samples from boxes 62–65 represented hard, laminated siltstone rocks situated between 245 and 260 m below ground level.

The timing of Wombat kimberlite emplacement is not directly known. However, zircon crystals recovered from a tephra layer within the Wombat lacustrine fill yielded a laser-ablation inductively-coupled-plasma mass-spectrometry (LA-ICP-MS) $^{206}$Pb/$^{238}$U date of 82.36 ± 0.44 Ma (Buryak, 2020), giving an early Campanian age for the Wombat lacustrine sequence.

## 3. Materials and methods

Rock samples from the Gleichen Pond (GP) outcrop, representing the Battle Formation, and mudstone and siltstone from the CH93- 29 core taken from the Wombat locality, were prepared for microscopy and examined for microfossil remains using light microscopy (LM) and/or scanning electron microscopy (SEM). Although all samples taken along the vertical transect at the Gleichen pond (GP) outcrop were examined, only results from GP10, GP11 and GP13 where raphid pennate diatom specimens were confirmed are reported in this study (Fig. 2A). These samples are situated between 2 and 2.6 m from the base of the formation along the 3 m transect. The ten samples taken from the Wombat core and investigated as part of this investigation were from rocks removed from box 50 and boxes 62–65 (Fig. 2B).

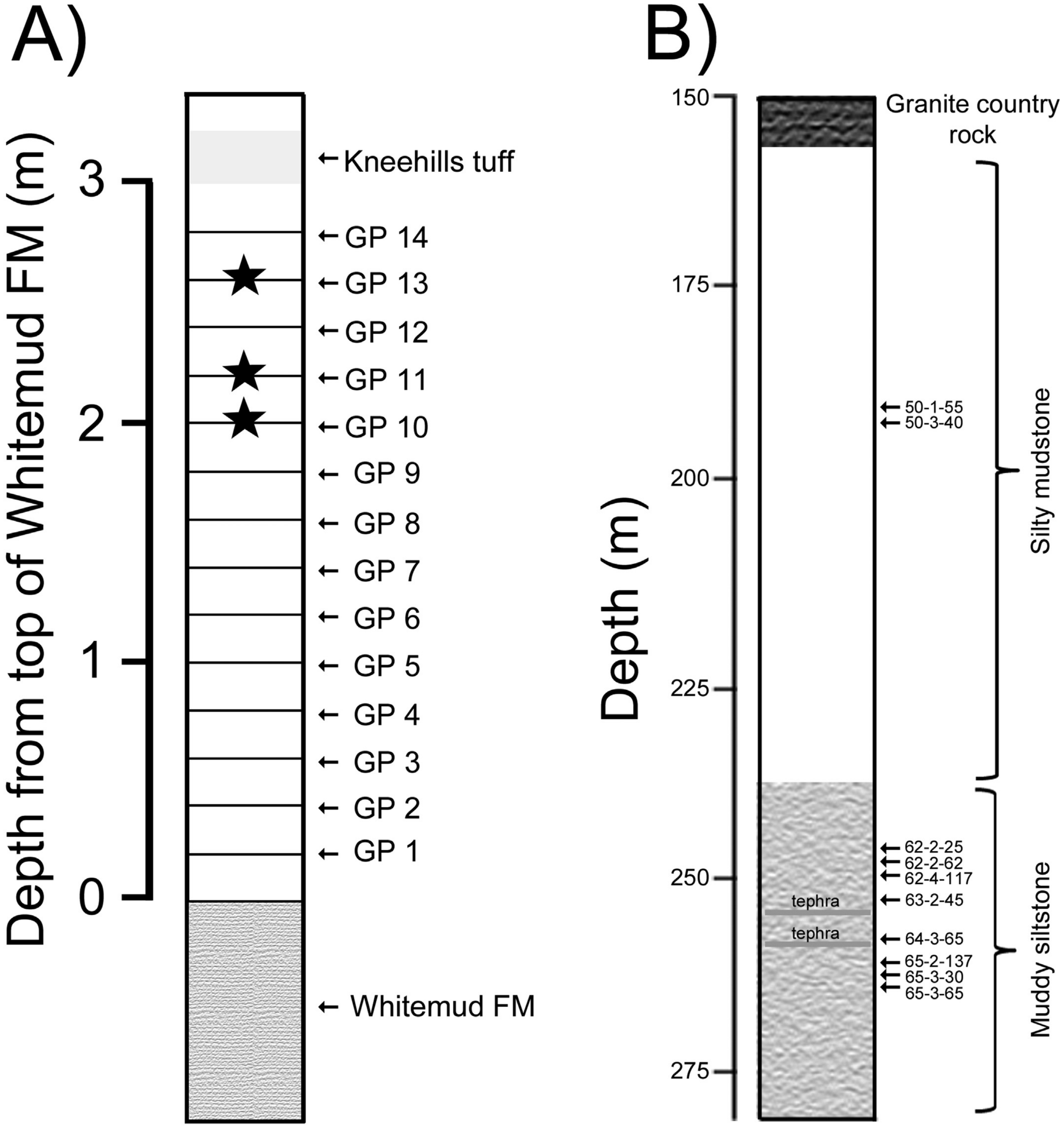


**Fig. 2.** Stratigraphic profiles of A) the Gleichen Pond (GP) outcrop of the Battle Formation, and B) the CH93-29 core taken from the Wombat fossil locality. The locations of the 14 samples taken from the GP outcrop are noted, and the positions of the Whitemud Formation and the Kneehills tuff depicted. The samples containing specimens of *Adlafia wolfeii* sp. nov. are labeled with a star. Locations of the ten strata from the Wombat core examined in this study are given.

The institutional repository of all studied material is the Canadian Museum of Nature (CMN), and the museum numbers (CANA) corresponding to the archived specimens are as follows: Battle GP13 (CANA 129313); W62-2-25 (CANA 129556); W62-2-62 (CANA 129557); W62-4-117 (CANA 129558); W63-2-45 (CANA 129559); W64-3-65 (CANA 129560); W65-2-137 (CANA 129561); W65-3-30 (CANA 129562); W65-3-65 (CANA 129563); W50-1-55 (CANA 129564) and W50-3-40 (CANA 129565).

Rock material used for LM and SEM analysis was washed in distilled water, fractured, and subsamples chipped from the freshly fractured surfaces. This process was repeated for 2–5 rock samples and the chips (100–500 mg of rock material) placed into a clean beaker. Each sample was then oxidized with 30% $H_2O_2$ under low heat for a minimum of an hour. For most samples, fresh $H_2O_2$ was then added and heated for an additional hour. The oxidized slurry was washed five times with distilled water using repeated centrifugation, the final pellet diluted to 15 ml, and stored in a glass vial. A new set of glassware, supplies and water bath were dedicated to each fossil locality for sample preparation.

For LM observation, aliquots of each sample were air dried onto glass coverslips, mounted onto glass slides with Naphrax, and examined at ×1000 magnification using Differential Interference Contrast optics with a Leica DMR compound microscope (N.A. 1.4) fitted with a Zeiss Axiocam 506 color camera. For SEM observation, aliquots of each slurry were air dried onto heavy duty aluminum foil, trimmed, and attached to aluminum stubs with Apiezon® wax. Samples were coated with a mixture of gold and palladium for 2 min with a Polaron Model E sputter coater, and examined with an FEI Nova NanoSEM 450 FESEM. Measurements of specimens were derived from electron micrographs (Supplemental material).

Three to four prepared slides and a minimum of two to three SEM preparations were thoroughly examined for each sample. Diatom specimens, especially broken pieces, were commonly found with LM and SEM for the Battle samples. However, because most diatom specimens from the Wombat samples remained affixed to rock fragments (Siver et al., 2016), they were observed primarily with SEM. Samples from each stratum taken from the Battle locality were prepared and studied independently by both P.A. Siver and M. I. Velez, and yielded similar results. Specimens of *Eunotia* uncovered in the Wombat core and representing the same species were each assigned a taxon number specific to this core.

## 4. Results

### 4.1. Records of early freshwater raphe-bearing diatoms

Raphe-bearing diatom records from 12 Eocene or older fossil localities are presented or reviewed as part of this study (Table 1). In addition to new findings from the Battle Formation, and the Wombat locality, results from ten previous investigations are summarized below.

The four oldest records of raphid pennate diatoms are from the Late Cretaceous, range in age from 82 to 65 Ma, and represent remains from the Wombat locality (Canada), Tarahumara Formation (Mexico), the Battle Formation (Canada), and the Deccan Intertrappean beds (India) (Table 1). Except for the Wombat locality, preservation of the specimens from the other three Cretaceous sites is fair to poor. The age of the specimens from the Tarahumara Formation, and whether the site was freshwater or brackish, has been questioned (Sims et al., 2006). In addition, because of the chertification of the Tarahumara specimens, the generic identifications are tentative, and presence of a raphe is assumed on the "*Amphora*-like" specimens. It is possible that the *Amphora*-like specimen is actually *Eunotia* or another genus. The identifications of *Nitzschia* Hassall and *Planothidium* Round & Bukhtiyarova within rhizopod tests uncovered from the Deccan Intertrappean beds appear correct, although only one image of *Planothidium* depicting the non-raphe bearing valve is presented. The Battle Formation specimens of *Adlafia* (see below) clearly possess a raphe. It is unclear if the two records from Russian localities, made over 100 years ago, are truly Paleocene (Ross and Sims, 1985; Sims et al., 2006). Except for the *Eunotia* specimens uncovered in the Wombat core and presented below, all of the other Cretaceous-Paleocene records represent raphe-bearing diatoms in the Bacillariophycidae.

Five localities spanning a 12 million year period from the early to middle Eocene (52–44 Ma) contain specimens with good to excellent preservation. All five localities harbored raphe-bearing genera in the Bacillariophycidae, and Eunotiophycidae taxa were uncovered from four of the sites (Table 1). In addition to *Eunotia*, multiple species of the Eunotiophycidae genus *Actinella* Lewis were also found to be abundant in the Giraffe Pipe material. The Wagon Bed Formation was especially productive yielding a new araphid genus (*Ambistria* Lohman & Andrews), many new species, and representatives from five Bacillariophycidae genera. We have examined material from Eocene-aged Eckfeld maar deposits and confirm remains of *Pinnularia* Ehrenberg and *Eunotia* from this locality.

Of the two youngest fossil localities from the late Eocene, the Florissant Formation in Colorado and the Oamaru locality in New Zealand, the former site yielded an impressive 18 raphe-bearing genera, all of which can be linked to modern congeners. Based on the Florissant Formation locality, it is clear that many of the modern lineages containing raphe-bearing genera had evolved by the end of the Eocene. Although the Oamaru locality represents a nearshore marine locality, the deposits are believed to contain freshwater diatoms washed in from land-based sites (Novitski and Kociolek, 2005; Williams and Reid, 2006).

### 4.2. Raphe-bearing taxon from the Battle Formation

A definitive raphe-bearing naviculoid species from the Battle Formation and representing the genus *Adlafia* is herein described from samples taken from the GP10, GP11 and GP13 strata:
Systematic position:

Kingdom Chromista T. Cavalier-Smith 1981
Phylum Bacillariophyta G. Karsten 1928
Subphylum Bacillariophytina Medlin & Kaczmarska 2004
Class Bacillariophyceae E. Haeckel 1878
Subclass Bacillariophycidae D.G. Mann 1990 (In: Round et al., 1990)
Order Cymbellales D.G. Mann 1990 (In: Round et al., 1990)
Family Anomoeoneidaceae D.G. Mann 1990 (In: Round et al., 1990)

Genus *Adlafia* G. Moser, H. Lange-Bertalot & D. Metzeltin 1998
Type species: *A. muscora* (Kociolek & Reviers) G. Moser, H. Lange-Bertalot & D. Metzeltin

*Adlafia wolfeii* sp. nov P. A. Siver & M. I.Velez (Figs. 3A–I; 4A–F).

*Description*. Valves are linear with straight to strongly triundulate margins, protracted and capitate to rostrate apices, and a centrally-positioned raphe (Figs. 3A–E; 4D–F). The margin undulations are of equal diameter (Figs. 3A–E; 4D). Valves range in size (including the undulation) from 18 to 35 μm × 4.5–8.1 μm. The raphe is straight, filiform, with circular to teardrop-shaped proximal fissures that deflect very slightly to the same side of the valve (Figs. 3B, 4A–B), and distal fissures that are strongly deflected to the opposite side of the valve from the proximal ends (Fig. 4E–F). The deflection of the distal raphe fissures is approximately 1.5–3 μm from the apex, and the proximal raphe ends are approximately 2 μm apart. Central area is small, circular, and lacks a fascia. The valve margin forms a distinct right angle with the mantle (Fig. 4D, F). Striae are uniseriate and composed of circular-shaped areolae that are continuous onto the mantle, and around the apices. Based on observations made with LM, the frustules are intact on most specimens, and the girdle is short and likely composed of two copulae. Internal structure not observed with SEM.

*Holotype*. Circled specimen on slide labeled "Battle GP13 - A" and deposited at the Canadian Museum of Nature as CANA 129313. Specimen is illustrated in Fig. 3A–E.

*Type material*. Mudstone rock from section GP 13 of the Battle Formation collected by P.A. Siver and M. I.Velez in September, 2017.

**Table 1**
Fossil records of raphid diatoms from twelve sites from the upper Eocene to the Upper Cretaceous.

| Location | Horizon | Reported raphid genera | Notes | Key references |
|---|---|---|---|---|
| Oamaru, New Zealand | upper Eocene (32–35 Ma) | *Amphora, Eunotia, Colliculoamphora* | Nearshore marine sediments and assemblage; terrestrial contaminants | Novitski and Kociolek (2005); Williams and Reid (2006) |
| Florissant Formation, Colorado, USA | upper Eocene (34 Ma) | *Achnanthidium, Adlafia, Amphora, Cymbella, Diadesmis, Epithemia, Eunotia, Planothidium, Rhoicosphenia, Gomphonema, Gomphoneis, Frustulia, Pinnularia, Platessa, Navicula, Nitzschia, Rhopalodia, Stauroneis* | Fissile paper shales with highly variable preservation | Benson et al. (2012), Benson and Kociolek (2012) |
| Eckfeld Maar, Germany | middle Eocene (44 Ma) | *Eunotia, Pinnularia* | Excellent preservation in some laminate facies | Lutz et al. (2010), this study |
| Dewey Beds, Idaho, USA | middle Eocene (45 Ma) | *Eunotia, Frustulia, Gomphoneis*? | Examination of slides archived at California Academy of Science | Benson et al. (2012) |
| Wagon Bed Formation, Wyoming, USA | lower–middle Eocene (48 Ma) | *Pinnularia, Stauroneis, Navicula, Nitzschia, Brachysira* (reported as *Anomoeoneis*) | Best preservation occurs in matrix between articulated bivalve shells that are less readily silicified | Lohman and Andrews (1968) |
| Giraffe kimberlite maar, subarctic Canada | lower–middle Eocene (48 Ma) | *Eunotia*, *Actinella*, *Nupela* and *Pinnularia* | Excellent preservation in both laminated and massive facies; numerous siliceous organisms | Siver and Wolfe (2007),<br>Siver et al. (2010),<br>Siver et al. (2015),<br>Souffreau et al. (2011) |
| Horsefly, British Columbia, Canada | lower Eocene (52 Ma) | *Frustulia, Eunotia* | Early Eocene, good preservation | Benson et al. (2012) |
| Archangelsk and Ananino, Russia | Paleocene ? | *Navicula?, Lyrella* | Marine, originally collected by Witt (1886) and Pantocsek (1889) | Ross and Sims (1985),<br>Sims et al. (2006) |
| Lameta Formation, India | Terminal Cretaceous | *Nitzschia, Planothidium* | Internal contents of arcellacean testate amoebae | Singh et al. (2006) |
| Battle Formation, Canada | Upper Cretaceous (67 Ma) | *Adlafia* | poor preservation, but raphe conclusively confirmed | This study |
| Tarahumara Formation, Mexico | Upper Cretaceous (70 Ma) | Putative naviculoid morphotype with affinity to *Amphora*. Raphe not observed | Poor preservation, chertification prevents examination of detail | Chacón-Baca et al. (2002);<br>Beraldi-Campesi et al., 2004 |
| Wombat kimberlite maar, subarctic Canada | Upper Cretaceous (82 Ma) | *Eunotia* | Excellent preservation in mudstones | This study |

Material deposited at the Canadian Museum of Nature as CANA 129313.

*Epithet*. The species is named in honor of Dr. Alexander P. Wolfe, paleontologist, diatomist, long-time colleague, collaborator, and friend.

*Remarks*. A few specimens had valve margins that were straight and parallel, with only slight undulations (Figs. 3F–I; 4A–B). Based on a specimen depicted earlier by Siver et al. (2018) (Fig. 5D), the axial area is narrow. Although specimens of *A. wolfeii* were routinely observed in samples from GP10, GP11 and GP13, most were broken valves or frustules, and whole specimens were more difficult to find. In addition to *A. wolfeii*, most of the araphid pennate diatom taxa reported in older rocks from the Battle Formation by Siver et al. (2018), as well as an additional *Aulacoseira* species, were also found in GP 10, GP 11 and GP 13.

### 4.3. New records of Eunotia from the Wombat locality

Definitive *Eunotia* specimens (Figs 5A–G) have been uncovered in eight strata from the Wombat core spanning 19.9 m (62-2-25, 62-2-62, 62-4-117, 63-2-45, 64-3-65, 65-2-137, 65-3-30 and 65-3-65), and potential remains from two additional and younger strata (50-1-55 and 50-3-40). The eight older samples were taken from muddy siltstone rocks, and the two younger samples from silty mudstone rocks. The differences in the ages between the strata are not known, but the assumption is the entire sequence represents thousands of years. Collectively, whole valves or fragments of valves from at least five different *Eunotia* species have been found. Even though insufficient numbers of specimens have been uncovered to justify formal descriptions at this time, preliminary details of the five species, each referred to with a unique taxon number, are as follows. Taxon #1 is a small, lunate-shaped diatom with a smooth and convex dorsal side and a relatively straight ventral surface, ranging in length from approximately 20–27 μm, and found in strata from box 62 (Fig. 5A; 62-4-117). Valve face striae are widely spaced, with only 9–10 per 10 μm. The stria density becomes 2–3 times as dense on both the dorsal and ventral mantles. The valves are reinforced with a thickening along the margin of the mantle at the apices. Details of the raphe were not observed. Taxon #2, uncovered from strata in boxes 62 and 63, represents another small species, approximately 25–30 μm long, with a simple lunate shape (Fig. 5B, 63-2-45; and C–D, 62-2-25). It is similar to Taxon #1 with respect to size and shape, but has a much greater stria density on the valve face. Each raphe slit originates on the mantle about 1/3 the distance from the apex, and both the proximal and distal ends terminate as small circular pores. Interestingly, one or both distal raphe ends bend up onto the valve face, and then deflect further towards the valve apex (Fig. 5B, D). Only a few partial remains of valves representing Taxon #3 have been found from sample 62-2-62 (Fig. 5F). Valves appear to be more linear-shaped with a straight ventral surface, possible dorsal undulations, and an increase in stria density at the apices. A helictoglossa and single rimoportula were observed (arrow, Fig. 5F). Taxon #4 possesses small, lunate-shaped valves and broad rounded apices (Fig. 5E; 64-3-65). This taxon, found only in sample 64-3-65, is approximately 18 × 4 μm with an even diameter across the valve. There are approximately 20 striae per 10 μm. Specimens were poorly preserved and details of the raphe system are lacking. Taxon #5 consists only of fragments and is the only representative in the younger samples from box 50 (Fig. 5G; 50-3-40). The fragments display a distinct right angle between the valve face and mantle, and a stria density ranging from 12 to 15 per 10 μm. Reduced raphe slits are observed, but it is not clear if these are on the mantle or valve face. The enlarged diameters of areola on specimens of taxon #5 indicate some dissolution.

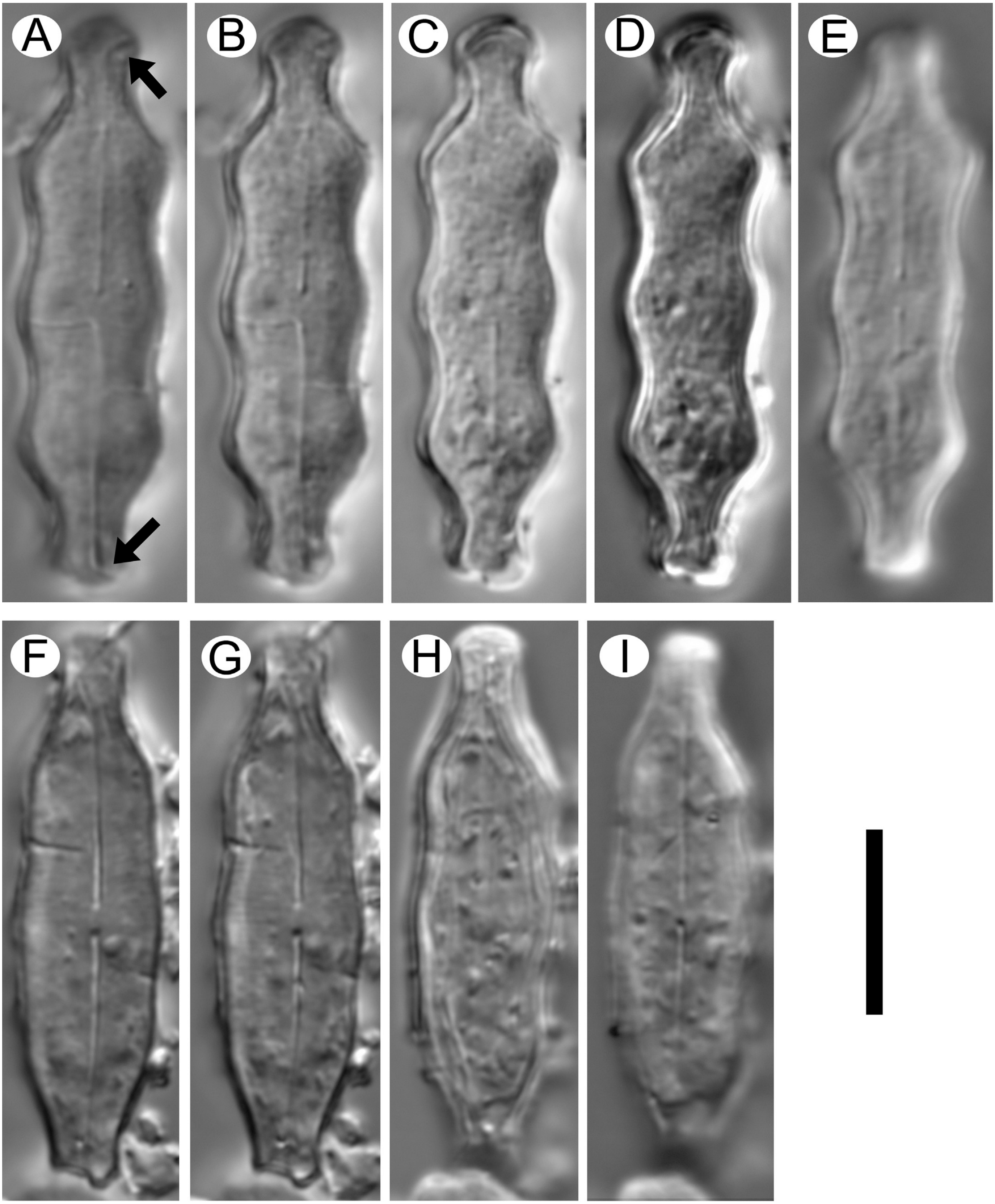
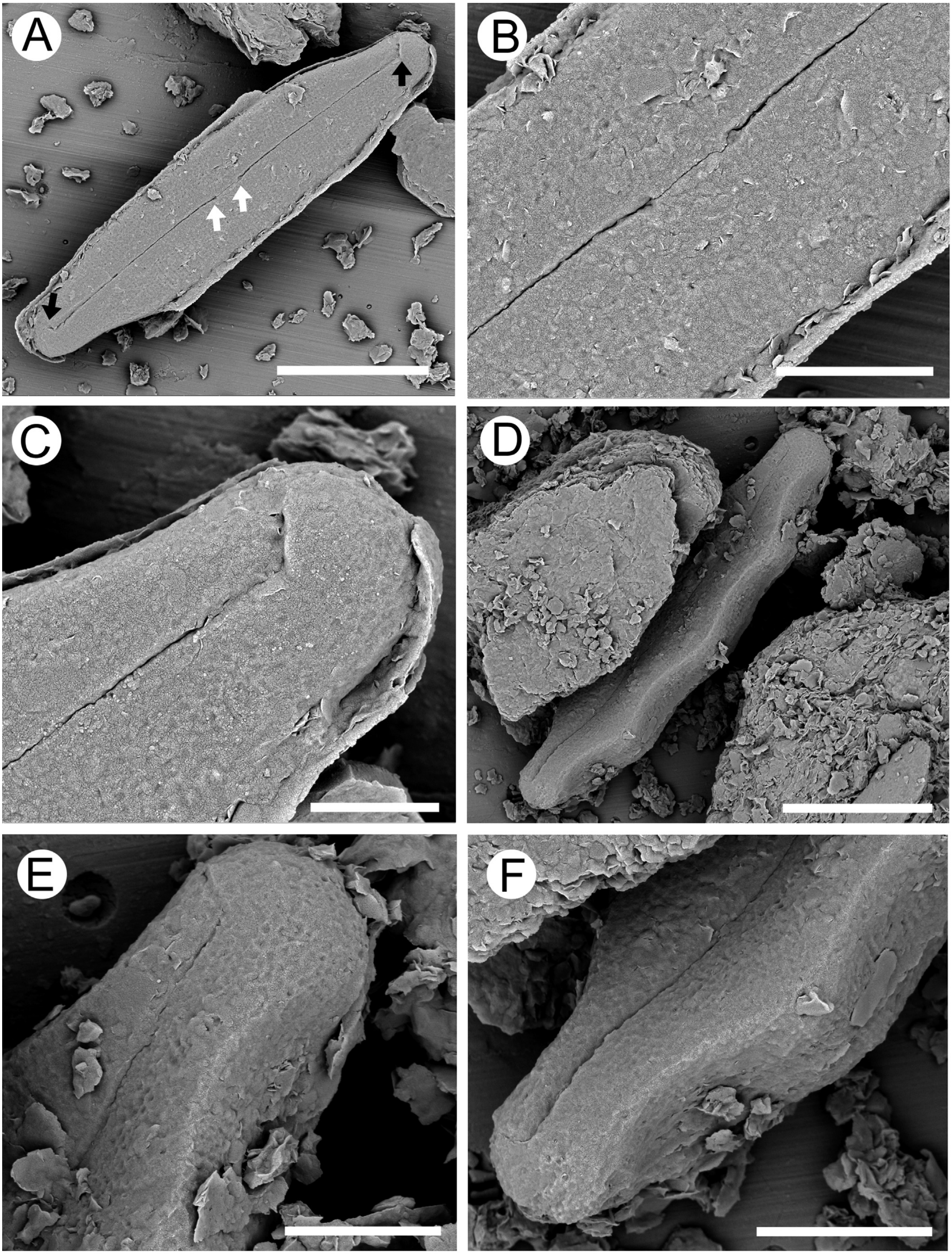


**Fig. 3.** (CANA 129313). Light micrographs depicting through focus images for two specimens of *Adlafia wolfeii* sp. nov. from the Cretaceous Battle Formation. The specimen in A–E represents the type specimen and illustrates the typical triundulate valve margins, capitate apices, slightly deflected proximal raphe fissures, and strongly deflected distal fissures (black arrows on A). The specimen in F–I has less wavy margins and rostrate apices. Scale bar = 10 μm.


A
B
C
D
E
F

## 5. Discussion

The oldest known fossil freshwater localities that definitively harbor diatoms are from the Late Cretaceous, and include the Battle Formation in western Canada (~67 Ma, Siver et al., 2018; this paper), the Deccan Intertrappean beds in India (end of the Cretaceous, Ambwani et al., 2003; Singh et al., 2006), the Tarahumara Formation in Mexico (ca. 70 Ma, Beraldi-Campesi et al., 2004; Chacón-Baca et al., 2002), and now the Wombat locality in subarctic Canada (82 Ma, this paper). Although some of the specimens from the Tarahumara site are most likely freshwater taxa (e.g. *Tabellaria* Ehrenberg ex. Kützing), Sims et al. (2006) questioned whether others were truly freshwater and suggested the site may represent a brackish environment. Nonetheless, diatoms had clearly colonized freshwater habitats by the Late Cretaceous. Soon thereafter, an increasing number of fossil records from freshwater Paleogene sites signal a radiation in diversity and types of environments inhabited by diatoms (e.g. Lohman and Andrews, 1968; Bullwinkel and Riegel, 2001; Wolfe et al., 2006; Wolfe and Siver, 2009; Siver et al., 2010; Benson and Kociolek, 2012; Siver et al., 2015). More interesting, the available evidence points to the idea that the raphe likely originated in a freshwater environment.

Development of the raphe, and with it the capacity to move across surfaces, represents perhaps the most significant event in the evolution of the diatoms (Round et al., 1990; Sims et al., 2006). Motility yielded the ability to move away from disturbance and avoid being buried by sediment, aided the cell in finding a potential mate for sexual reproduction, allowed specimens to move between microhabitats, and increased the number of habitats that could potentially be colonized (Kooistra et al., 2007; Nakov et al., 2018). With the development of the raphe, coupled with many subsequent tweaks and modifications, came a radiation of new diatom morphotypes resulting in the taxon-rich Bacillariophyceae. Indeed, high rates of species diversification have been found among raphe bearing forms (Nakov et al., 2018). Although Cenozoic diversity in marine diatoms has been linked to climate change (Lazarus et al., 2014), evolution of the raphe was likely a major factor driving the Cenozoic diversification of freshwater forms. By the end of the Eocene most of the lineages of raphe-bearing diatoms had already evolved (Sims et al., 2006).

Siver et al. (2018) first reported on freshwater diatoms in sections GP5—GP7 from the same locality of the Battle Formation investigated in the current study. Their findings included what is the oldest known record for *Aulacoseira* Thwaites, and seven pennate taxa. The description of the pennate species identified as morphotype #2 in that study (see Fig. 4A—C and fig 4D in Siver et al., 2018), clearly match specimens of *Adlafia wolfeii*, especially with respect to valve outline and overall dimensions. Given the poor preservation of specimens in the earlier work from lower strata in the formation, Siver et al. (2018) were not able to confirm the presence of a raphe on morphotype #2. Description of a second taxon, morphotype #6, made by Siver et al. (2018, Figs. 4D and 5D) was similar to their morphotype #2, but with less undulated margins and the presence of a linear feature along the sternum. The linear feature was reported to possibly represent a raphe, but again due to preservation quality this could not be confirmed. Given the new information, we conclude that morphotypes #2 and #6 reported in the earlier study represent *Adlafia wolfeii*. The new observations presented in the current study, based on specimens from younger strata within the Battle Formation (e.g. GP 10, GP11 and GP 13), and with slightly better preservation, confirm that freshwater raphe-bearing naviculoid species had evolved by the Late Cretaceous. Because the presence of a raphe on specimens suggested to represent *Amphora* Ehrenberg ex. Kützing from the Tarahumara Formation (Chacón-Baca et al., 2002) was never confirmed, *Adlafia wolfeii* may represent the oldest known Bacillariophycidae diatom confirmed to possess a raphe, and the ~67 Ma age is inching closer to the Cretaceous date for evolution of the raphe estimated by Nakov et al. (2018).

According to the molecular-based phylogeny presented by Nakov et al. (2018), the genus *Adlafia* is approximately 45Ma, and the age of the common ancestor with its sister lineage around 55 Ma. Because the analysis by Nakov et al. (2018) only included one species of *Adlafia*, the 45 Ma represents a minimum estimate for the genus. If additional *Adlafia* species had been included, the minimum age for the genus may have been older. Given our findings, *Adlafia* evolved at least 20 Ma earlier, and this new age constraint provides yet another valuable marker for use in future works.

The genus *Adlafia*, described by Moser, Lange-Bertalot and Metzeltin (1998), contains some species originally placed within the genus *Navicula* Bory. Species of *Adlafia* are generally small and possess a filiform raphe with simple proximal raphe endings that are slightly deflected in the same direction, distal raphe fissures strongly bent in the same direction, but opposite those of the proximal ends, uniseriate striae consisting of relatively large areolae that are covered externally with hymens, and a girdle consisting of two open and perforated copulae (Moser, Lange-Bertalot and Metzeltin, 1998; Van de Vijver et al., 2019). Except for details of the external areolae coverings, the primary diagnostic characters were observed on the Battle taxon, confirming its placement in *Adlafia*.

Comparing to contemporary diatoms, *Adlafia wolfeii* is most closely related to *Adlafia tenuis* Van de Vijver & Goeyers. This taxon was originally described as *Navicula tenuis* Krasske, later as *N. tridentula* v. *tenuis* Lange-Bertalot & Willmann, and eventually transferred to *Adlafia* (Van de Vijver et al., 2019). Both species are relatively small, with linear valves possessing triundulate margins, protracted capitate apices, a raphe with slightly deflected proximal fissures and strongly deflected distal fissures, and uniseriate striae with large circular areolae. In addition, the girdle of *A. wolfeii* specimens is shallow and appears to consist of no more than two bands. The two species differ in a number of key characters. Valves of *A. wolfeii* are longer and wider than those of *A. tenuis*. The valve margins on *A. wolfeii* range from being straight to triundulate, whereas those of *A. tenuis* are always undulate. The proximal raphe fissures are large, prominent and circular to teardrop-shaped on *A. wolfeii* valves, whereas on *A. tenuis* valves they are simple and non-distinct. The raphe branches on *A. wolfeii* valves are straight and not undulate as they are on *A. tenuis* specimens. Lastly, based on available observations, the axial area appears to be narrower on *A. wolfeii* valves.

Morphological and molecular data support development of the raphid diatoms from araphid ancestors (Sims et al., 2006; Sorhannus, 2007; Medlin, 2015; Nakov et al., 2018). Further, based on available fossil evidence as presented in the current study, the earliest raphe-bearing diatoms are known exclusively from freshwater environments, implying that this structure did not evolve in a marine setting. An unsettled question is whether the initial raphe was similar to the eunotioid design consisting of a pair of short

**Fig. 4.** (CANA 129313). SEM micrographs of *Adlafia wolfeii* sp. nov. from the Cretaceous Battle Formation. A—C depicts a specimen with straight valve margins and rostrate apices. The proximal raphe fissures (white arrows on A; B) terminate as small circular pores that are slightly deflected to one side of the valve. The distal raphe fissures (black arrows on A) are strongly deflected towards the opposite side of the valve. D—F depicts a specimen with strongly triundulate margins. The strongly deflected distal raphe fissures are illustrated in E—F, and outlines of relatively large and circular areolae are best observed in E. Scale bars = 2 μm (C), 3 μm (B, E), 4 μm (F) and 10 μm (A and D).

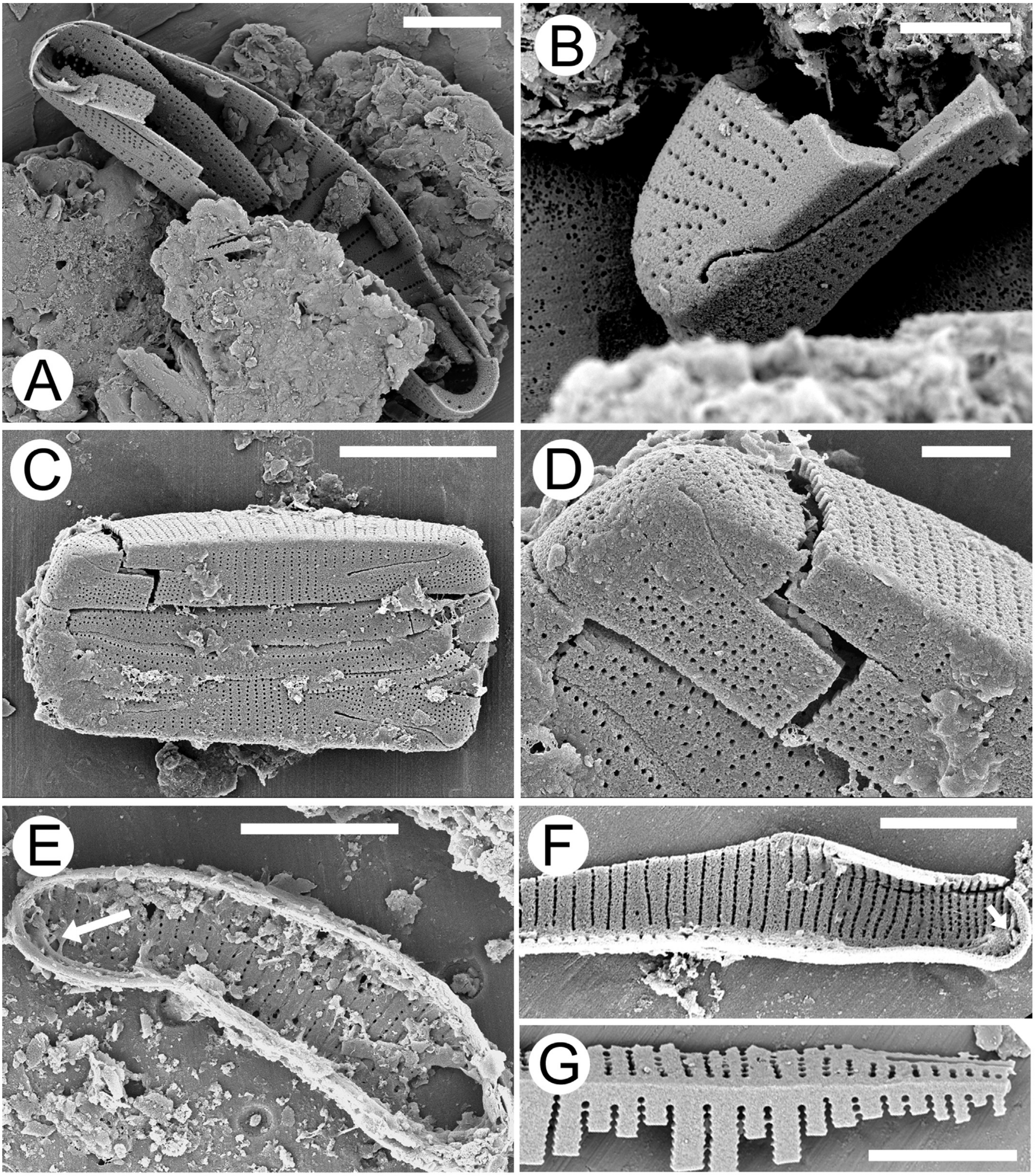


**Fig. 5.** SEM micrographs of *Eunotia specimens* from the Cretaceous Wombat locality. A (CANA 129558). Taxon #1. Note the widely-spaced striae on the valve face, and more densely-spaced striae on the mantle. B (CANA 129559) and C–D (CANA 129556). Specimens of taxon #2, including a whole frustule depicting the arrangement of the raphe slits on the mantle. Note the forward-projecting distal raphe fissures and the similar stria density on both the valve face and mantle. E (CANA 129560). A small and lunate-shaped valve of taxon #4. The arrow depicts the position of the helictoglossum. F (CANA 129557). A portion of the linear-shaped valve of taxon #3, depicting a straight ventral surface, possible dorsal undulations, an increase in stria density at the valve apices, and the positions of both the helictoglossa and rimoportulae (white arrow). G (CANA 129565). Taxon #5. A portion of a valve showing a distinct right angle between the valve face and mantle, and the remains of a raphe slit. Note partial dissolution associated with the areolae. Scale bars = 2 μm (B, D), 5 μm (A, E-G) and 10 μm (C).

apical slits on the valve margin, later migrating to and becoming incorporated into the central sternum, and with subsequent elongation and elaboration of the slits. Further, the presence of rimoportulae led Berg (1948) to propose that eunotioid diatoms evolved from rimoportula-bearing araphid diatoms, and Kolbe (1956) and Hasle (1974) hypothesized that the eunotioid raphe slits evolved from elongation or expansion of apical rimoportulae. This hypothesis was further advanced by Mann (1984).

An alternative hypotheses to that proposed by Mann (1984) and others, is that the eunotioid raphe type was derived within the raphid clade, moving from a central position on the valve face to a marginal position on the mantle, possibly in response to development of an asymmetric lunate shape. A centrally positioned raphe on a lunate-shaped valve would not be an energetically favorable design for movement. Instead, placement of shorter raphe slits on apical ends of the mantle, coupled with a concave ventral surface, would reduce drag during movement across surfaces. This alternative hypothesis means that eunotiod diatoms would hold a derived position within the raphid clade, a finding supported by Medlin and Kaczmarska (2004), Sorhannus (2007), and more recently by Nakov et al. (2018). If the eunotioids are indeed a derived group, and because *Eunotia* is now documented to be at least 82 Ma old based on findings from the Wombat locality, the evolution of the raphe likely occurred at least in the Early Cretaceous if not earlier.

It is interesting that multiple molecular studies align the eunotioids with species belonging to *Nitzschia* and *Achnanthes* Bory sensu lato (Medlin and Kaczmarska, 2004; Sorhannus, 2004), genera considered to be quite different from the eunotioids (Round et al., 1990). Because eunotioid species possess rimoportulae, this association seems even more questionable if indeed the eunotioids are a derived lineage within the raphid clade (Medlin, 2016). If this were the case, the rimoportulae would have most likely evolved at least twice. However, if the clade containing eunotioids, *Nitzschia* and *Achnanthes* has an older common ancestor with other raphe-bearing diatoms, then perhaps the presence of rimoportulae among the eunotioid lineage is less problematic. Further, it is interesting that in addition to *Eunotia*, *Nitzschia* and *Planothidium* (in the Achnanthales) are among the oldest known raphe-bearing diatom fossils (Singh et al., 2006).

Most of the *Eunotia* specimens uncovered from the Wombat locality possess features that clearly place them into this genus, including overall valve shape and symmetry, short raphe slits on the mantle, and the presence of rimoportula. Several characters of the Wombat specimens have not been observed, or are rare, on modern species and are worth noting. First, although the raphe on many modern species bends from the mantle up onto the valve face and then recurves backwards towards the valve center, none have distal raphe fissures that bend forward towards the apex as observed on Wombat taxon #2. In addition, the shapes of the distal raphe ends on the same valve of some taxon #2 specimens differ, another feature not observed on modern species. Lastly, the wide disparity in stria density between the valve face and mantle displayed on Wombat taxon #1 is rare or lacking on modern species. It will be interesting to note if these characters are eventually found on additional fossil forms.

Even with the findings reported here, the potential that freshwater diatoms, including raphe-bearing forms, are considerably older than Cretaceous cannot be ruled out. In a thorough review, Siemińska (2000) summarized earlier reports of freshwater fossil diatoms uncovered from Mesozoic, Paleozoic and even Proterozoic rocks. Many of these earlier studies depicted diatoms that can be clearly linked to modern taxa. For example, Heinrich (1913) and Zanon (1929) reported numerous raphe-bearing diatoms from Upper Triassic rocks that can be readily linked to modern lineages. These included, but are not limited to, species of *Pinnularia*, *Epithemia* Kützing, *Cymbella* C. Agardh, *Eunotia*, *Nitzschia* and *Surirella* Turpin. Castracane (1876) reported finding diatom remains, including raphe-bearing forms, in burned coal ash, and Grüss (1928) uncovered nitzschioid diatoms in Devonian coal schist. Pia (1931) attributed most of these earlier findings to contamination and in some cases also incorrect age estimates. Even more intriguing are the findings of Siemińska and Kwiecińska (2000) who uncovered diatom specimens from Proterozoic black marbles. Although it is questionable if some of the specimens these authors illustrate from the black marbles represent diatoms, others are clearly fragments of valves, including ones of nitzschioid specimens. More recently, Farooqui et al. (2015) reported freshwater diatoms from the Early Permian (295 Ma) Manjir Formation in India, that also included nitzschioid forms. What is especially interesting is that many of the diatoms found in these ancient rocks include species with what are thought to be advanced and complex raphes, including taxa belonging to *Pinnularia* and nitzschioid genera. If these fossil records are correct, and we can provide no additional information to prove or disprove them, then the age of the diatoms inferred from most molecular analyses underestimates the origin of the group.

## 6. Conclusions

In summary, raphe-bearing pennate diatoms representing both the naviculoid and eunotioid types are confirmed from the Late Cretaceous. Both raphe types, along with many of the diatom lineages of Bacillariophyceae known today, are well represented in the fossil record by the Eocene. The taxa described from the Battle Formation and from the Wombat locality are among the oldest, if not the oldest, known specimens from the fossil record that possess a raphe. Available evidence at this time further supports a freshwater origin for the raphe. The geologic ages represented by *Adlafia wolfeii* sp. nov. at 67 Ma, and *Eunotia* at 82 Ma, will provide important new markers for aligning molecular-based phylogenies to geologic time.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cretres.2022.105456.