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March 11, 2011

Mr. David LaCaro, Environmental Scientist II
Regional Water Quality Control Board, Central Coast Region
895 Aerovista Pl., Ste. 101
San Luis Obispo, CA 93401-7906

Via E-Mail to dlacaro@waterboards.ca.gov

Re: Waste Discharge Requirements for the Los Osos Water Recycling Facility (Draft Order No. R3-2011-0001 & Draft MRP Order No. R3-2011-0001)

Dear Mr. LaCaro:

We submit these comments on behalf of our client, Citizens for a Sustainable Community (Citizens), for consideration by the Central Coast Regional Water Quality Control Board (CCRWQCB) in deciding whether to approve the waste discharge requirements (WDRs) proposed for the Los Osos Water Recycling Facility (LOWRF), a project of the County of San Luis Obispo (County).[1] For the reasons set forth below and based on the additional information contained in the attached addendum and exhibits, we request that before the CCRWQCB takes any action approving the draft WDRs, it order preparation and public circulation of a subsequent or supplemental environmental impact report (SEIR), and consider the information in that SEIR. Such a course of action is mandated by the California Environmental Quality Act (CEQA) (Pub. Res. Code § 21000 et seq.) and the State CEQA Guidelines (Guidelines) (Cal. Code Regs., tit. 14, § 15000 et seq.), among other things, due to new information of substantial importance that was not known -- indeed, did not exist -- and could not reasonably have been known and considered in 2009, when the County certified the environmental impact report (EIR) for the LOWRF project

[1] Please note that in the addendum attached to this letter, the LOWRF project is referred to as the Los Osos Wastewater Project (LOWWP) -- the project name used by the County.

(acting as lead agency under CEQA).[2] Furthermore, as proposed, the CCRWQCB's action on the project will violate CEQA for additional reasons and will also violate the Porter-Cologne Water Quality Control Act (Porter-Cologne Act) (Water Code § 13000 et seq.).

The CCRWQCB correctly notes that because it has discretionary review and approval power over the LOWRF project through its statutory responsibility to prepare WDRs, the CCRWQCB is a "responsible agency" within the meaning of CEQA for the purpose of environmental review of and action on the WDRs. (Guidelines, § 15381; see also § 21069.) An activity that involves the issuance to a person of a lease, permit, license, certificate, or other entitlement for use by one or more public agencies is subject to the CCRWQCB's CEQA review. (See § 21065, subd. (c).)

The CCRWQCB Must Prepare an SEIR and Comply with Its Duties as a Responsible Agency Under CEQA Before Approving the Draft WDRs.

Section 21166 and Guidelines sections 15162 and 15163 require the preparation of an SEIR when (1) substantial changes occur with respect to the circumstances under which a project is undertaken, such that revisions of the EIR are necessary due to the involvement of new significant environmental effects or a substantial increase in the severity of previously identified significant effects; or (2) new information of substantial importance which was not known and could not have been known with the exercise of reasonable diligence when the EIR was certified becomes available, showing that: (i) the project will have one or more significant effects not discussed in the EIR; (ii) significant effects previously examined will be substantially more severe than shown in the EIR; (iii) mitigation measures or alternatives previously found infeasible would in fact be feasible and would substantially reduce one or more significant effects of the project; or (iv) mitigation measures or alternatives considerably different from those analyzed in the previous EIR would substantially reduce one or more significant effects on the environment.

Substantial changes in physical baseline conditions under which the LOWRF project is undertaken have occurred and new information, including hydrologic data, has become available, meeting the criteria of section 21166 and Guidelines section 15162. The Los Osos Basin Plan Update publicly released in May 2010 (over seven months after certification of the EIR) by an interagency working group consisting of representatives from the County and the area's three major water purveyors shows rapidly accelerating seawater intrusion into the Los Osos Valley groundwater basin, the region's only drinking water source. As the CCRWQCB's staff report notes, according to the 2009 EIR, based on investigation data between 1985 and 2005, the rate of seawater intrusion into aquifer zones D and E are *60 and 54 feet per year*, respectively, but the May 2010 Basin Update now shows that the seawater wedge has extended into the same aquifer through fingers at the significantly higher rate of *700 feet per year*. The plume now reaches as far inland as a well near Los Osos Community Park and Los Osos Valley Rd. The saltwater movement data thus indicate a substantially more severe public health threat to water supply than previously known, and the County and the relevant water agencies no longer believe water demand is within safe yield (+/- 3,200 AFY) without measures counteracting this change.

[2] All unlabeled section (§) references in this letter are to CEQA provisions contained in the Public Resources Code.

Additional independent expert review of the Plan Update by Mr. Eugene Yates, a hydrogeologist, confirms that the recently discovered substantially accelerating seawater intrusion into the basin is an "extremely urgent" problem that requires urgent action, including 500 AFY of reduced pumping from the urban compartment. (Review dated August 3, 2010, at 1 [attachment #3 to our addendum].) Yates also recommended the review of a wide range of mitigation options to address changes in basin conditions, given that the LOWRF project, in conjunction with the increased pumping from the upper aquifer may induce seawater intrusion *in the upper aquifer*. Yates opined that accelerating seawater intrusion makes the LOWRF project's recycled water reuse program -- viewed as key mitigation for the LOWRF -- outdated, and may indeed make it nonviable.

The changed baseline conditions and new information are important as there is a direct nexus between seawater intrusion in the Los Osos groundwater basin, the rate thereof and the LOWRF project. As designed, the project, which includes the decommissioning of the onsite wastewater disposal systems in the prohibition zone, contributes to the environmental and public health effects (sodium contamination of the freshwater) of seawater intrusion by reducing groundwater recharge.[3] As noted in the Hopkins Groundwater Consultants report prepared for the 2009 EIR, project operation results in a net deficit of groundwater recharge and aquifer volume. The changed baseline conditions and new information concerning changes in the basin's freshwater/seawater interface thus are highly relevant to the CCRWQCB's environmental review of the LOWRF project, since with substantially accelerated landward saltwater movement into the drinking water reservoir, public health effects will now be substantially exacerbated, and the project will result in new, as yet unexamined, indirect and cumulative significant effects, including public health effects.[4] In this instance, for the CCRWQCB to "use its best efforts to find out and disclose all that it reasonably can," and conduct "thorough investigation," as it must under CEQA (Guidelines, §§ 15144, 15145), goes to the heart of the CCRWQCB's healthy watersheds vision. That vision is one of keeping groundwaters "near natural levels in quantity and quality for water supply purposes and for base flow for sustaining creek habitat, and migratory fish routes," while protecting watersheds "from hydromodification that adversely affects recharge area functions, or the stability of creeks' beds or banks." (CCRWQCB Dec. 11, 2011 Vision Message, at 1-2 & fn. 2 [<http://www.swrcb.ca.gov/centralcoast/publications_forms/publications/vision/docs/Agencies_ltr.pdf>, as of March 10, 2011].) Yet, as proposed, the WDRs include no "end-of-pipe" requirements or mitigations to reduce or

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[3] Loss of groundwater recharge lowers the water level (head) in the aquifer, which reduces the flow of freshwater to seawater. This flow reduction, in turn, allows inland movement of the seawater-freshwater interface. (See, e.g., U. S. Geological Survey, *Saltwater Intrusion in Los Angeles Area Coastal Aquifers-The Marine Connection* (2002 Fact Sheet) [<http://pubs.usgs.gov/fs/2002/fs030-02/>, as of March 10, 2010]; Frederick, *America's Water Supply: Status and Prospects for the Future* (Consequences, vol. 1, No. 1, Spring 1995) [<http://www.gcrio.org/CONSEQUENCES/spring95/Water.html>, as of March 10, 2011].)

[4] " 'Significant effect on the environment' means a substantial, or potentially substantial, adverse change in any of the physical conditions within the area affected by the project including land, air, water, minerals, flora, fauna, ambient noise, and objects of historic or aesthetic significance." (Guidelines, § 15382.)

avoid the combined direct, indirect and cumulative effects of the substantially changed groundwater baseline conditions and LOWRF project on groundwater quantity and quality.[5] Consistent with CEQA, we thus request public review in an SEIR of (1) the major change in physical baseline conditions affecting groundwater sustainability represented by the significantly increased saltwater movement into the basin; and (2) the combined environmental effects (including public health effects) of this change and the LOWRF project, including the relationship of the change to -- and the cumulative effects thereof on -- the changes in aquifer dynamics and freshwater storage that result from the implementation of the LOWRF project. SEIR assessment of the seawater intrusion impacts of the LOWRF project and of the comparative impacts of feasible wastewater treatment alternatives must be performed, and mitigation measures correlated to the actual severity of the impact (the real, anticipated salinity levels and their distribution) must be developed and implemented, based on specific, measurable, enforceable and verifiable performance standards. This SEIR assessment is critical to informed decisionmaking and public participation in the WDRs review and approval process. The post-2009 EIR information now available to the CCRWQCB negates the assumptions of hydrogeological and hydrochemical equilibria in baseline water conditions upon which the County EIR assessments and mitigations were based. Under those circumstances, should the CCRWQCB approve the WDRs, CEQA mandates preparation of an SEIR so the CCRWQCB and the public may be adequately informed of relevant, substantial or potentially substantial adverse environmental effects of the LOWRF project, considered in the *actual,* full hydrogeological and hydrochemical context, and based on *current* hydrogeological and hydrochemical data and environmental assessment using modeling that accounts for appropriate margins of safety.

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[5] "Clean groundwater" is one of the three central tenets of the CCRQQCB's healthy watersheds vision, which, as cogently stated on the CCRWQCB's Website, serves "to empower people to act on the important issues facing the Central Coast Region over the next 20 years." (<http://www.swrcb.ca.gov/centralcoast/publications_forms/ publications/vision/teams.shtml> [as of March 10, 2011].) Substantially accelerated seawater intrusion in the Los Osos Valley groundwater basin surely is one such issue. Because this hydrogeological process occurs in the area affected by the LOWRF project, it cannot be severed from the CCRWQCB's review of the WDRs. CEQA requires that physical baseline conditions affected by a project be disclosed in the environmental review document of the agency called upon to take permitting action for that project. As provided in Guidelines section 15125, subdivision (c): "Knowledge of the regional setting is critical to the assessment of environmental impacts. *Special emphasis* should be placed on environmental resources that are *rare or unique* to that region and would be affected by the project. The EIR [or, in this case, an SEIR] must demonstrate that the significant environmental impacts of the proposed project were adequately investigated and discussed and it must permit the significant effects of the project to be considered *in the full environmental context*." (Emphasis added.) Considering its high level beneficial use, the groundwater resource here surely is rare and unique to the region and the population that depends on it. (See also *County of Amador v. El Dorado County Water Agency* (1999) 76 Cal.App.4th 931, 954-955 [for adequate environmental assessment of fisheries, river habitat and recreational use impacts of lake water withdrawals associated with a water supply project, the permitting agency must disclose and evaluate relevant information on baseline lake levels, including rates of existing releases]; CEQA Guidelines, § 15126.2, subd. (a) [EIR should address, inter alia, the "health and safety problems caused by the physical changes, and other aspects of the resource base such as water … and public services"].)

Whenever an agency with discretionary approval authority over a permit or other entitlement for use for a project makes an approval decision, and the final EIR for the project fails to provide the environmental information disclosure mandated by CEQA, or is not supplemented to account for substantially changed circumstances in physical baseline conditions or to review significant new environmental information relevant to the project, the decision is a prejudicial abuse of discretion, and, therefore, a nullity. (See *Communities for a Better Environment v. City of Richmond* (2010) 184 Cal.App.4th 70, 88 [an agency's " 'ultimate decision of whether to approve a project, be that decision right or wrong, is a nullity if based upon an EIR that does not provide the decision-makers, and the public, with the information about the project that is required by CEQA' "]; *Mira Monte Homeowners Assn. v. County of Ventura* (1985) 165 Cal.App.3d 357, 361, 364-365 [failure to prepare an SEIR after resurvey of project site revealed significant new information, i.e., a greater seasonal wetlands impact than previously identified, nullified approval decision because it prevented consideration of "the full range and effectiveness of alternatives and mitigation measures"].)

Importantly, as a responsible CEQA agency, the CCRWQCB cannot defer to the County regarding whether or not to prepare an SEIR. By CEQA, the CCRWQCB must reach its own conclusions on whether and how to approve a project, based not only on its review of the environmental document prepared by the lead agency, but also any new information in an SEIR which the CCRWQCB must prepare where, as here, SEIR review is mandated. (See Guidelines, §§ 15020, 15052, 15096, subd. (a), 15162, 15163; *Save San Francisco Bay Assn. v. San Francisco Bay Conservation and Development Com.* (1992) 10 Cal.App.4th 908, 932; *id*. at 921-922 [responsible agency must "conduct its own analysis using its own special expertise" before "reach[ing] its own conclusions"].) As discussed in greater detail in the attached addendum, Guidelines section 15052 requires the CCRWQCB to step into the shoes of the County, and prepare (or contract preparation of) an SEIR prior to approving WDRs for the LOWRF project, because: (1) the prerequisites under section 21166 and Guidelines section 15162 for an SEIR are established; and (2) prior to the CCRWQCB's action, the lead agency (County) granted a final discretionary approval for the LOWRF project and the statute of limitations for challenging that approval has expired. (County approval occurred on September 29, 2009. The statute of limitations expired in 2009 -- 30 days after the posting of the County's notice of determination which gave public notice of the September 29, 2009 decision.)

Importantly, even absent -- yet especially because of the presence of -- the new physical baseline conditions and the new information summarized above, the CCRWQCB, as a responsible agency, must mitigate or avoid "the direct or indirect environmental effects of those parts of the project which it decides to carry out, finance, or approve." (Guidelines, § 15096, subd. (g)(1).) Also, "[w]hen an EIR has been prepared for a project, the Responsible Agency shall not approve the project as proposed if the agency finds any feasible alternative or feasible mitigation measures within its powers that would substantially lessen or avoid any significant effect the project would have on the environment." (Guidelines, § 15096, subd. (g)(2).) Finally, before approving the WDRs, the CCRWQCB must "make the findings required by [Guidelines] Section 15091 for each significant effect of the project and shall make the findings in [Guidelines] Section 15093 if necessary." (Guidelines, § 15096, subd. (h); see *Save San Francisco Bay Assn.*, 10 Cal.App.4th 908, 921-922, 932.)

The CCRWQCB thus clearly has a duty to mitigate or avoid the adverse impacts of the LOWRF project. The discretionary approval currently before the CCRWQCB involves mitigation for the LOWRF project's impacts to hydrology and water quality. The CCRWQCB staff report asserts that the CCRWQCB has no duty to make specific findings pursuant to Guidelines section 15096, subdivision (h), because the EIR did not identify any potentially significant impacts within the CCRWQCB's jurisdiction. This conclusion is unsupported, in light of both the new information discussed above, and previous information contained in the 2009 EIR itself, which identifies project impacts to federally regulated waters as potentially significant. Those impacts are within the CCRWQCB's jurisdiction. Specifically, construction of pipelines that will be suspended over two federally regulated creeks (Los Osos Creek and Warden Creek) will result in construction impacts to these creeks. As such, the County may have to obtain (a) a Clean Water Act section 401 Water Quality Certification from the CCRWQCB; and (b) a Clean Water Act section 404 permit from the U. S. Army Corps of Engineers, necessary for discharges of dredged or fill material into the federally regulated creeks. Both the staff report and the WDRs fail to describe and evaluate this potentially significant impact. Further, despite the EIR's identification of this potentially significant impact, the WDRs contain no findings regarding the project's effects on federally regulated waters, or potentially feasible alternatives or mitigation measures which would avoid or substantially lessen the effects.

In any event, given the changed water baseline conditions and the new information that has become available following certification of the 2009 EIR, the CCRWQCB must do more than prepare findings tracking the requirements of Guidelines section 15096, subdivision (h). It must prepare and circulate an SEIR, and explore and impose (following public review of the SEIR) feasible mitigation measures or a feasible alternative to the LOWRF project as proposed. Regardless of bureaucratic or political momentum behind the County's project, the next responsible agency must follow the law. It cannot simply defer to the lead agency without assuming its own CEQA duties, including its duties under section 21166 and Guidelines sections 15052, 15162 and 15096.

<u>The WDRs Unlawfully Defer Environmental Review Regarding Location and Impacts of Discharges from the LOWRF Project.</u>

The CCRWQCB staff report states that the County "included a list of areas proposed for disposal in its report of waste discharge application." (Staff report at 2; proposed WDRs at 2.) But these areas remain unspecified, and the list is not disclosed, let alone discussed in the staff report or the WDRs. The list appears to be nonbinding anyway. We are told, indeed, that "Details of the Discharger's reuse program are not yet available[,]" and that the reuse program has yet to be developed. (*Id*.)[6] The long and the short of it is: except for the Broderson leach field (Discharge

[6] The staff report and the proposed WDRs, by use of four separate bullet points, confusingly refer to four separate "discharge points," as follows:

> "• Discharge Point 1: Agricultural reuse irrigation at 25 different locations.
> "• Discharge Point 2: Broderson leach field.
> "• Discharge Point 3: Bayridge Estates leach field at 2 locations.
> "• Discharge Point 4: Urban reuse irrigation at 10 different locations."

Point 2) and (perhaps) the two locations of the Bayridge Estates leach field (Discharge Point 3), the CCRWQCB is being requested to approve WDRs for many unspecified discharge locations, that is, without knowing where much of the wastewater the discharges of which it must permit will be discharged.

This is a startling request, as is the gap in information regarding where wastewater discharge impacts will occur, and thus what their onsite and their offsite (runoff) effects will be. The informational gap precludes assessment of water quality impacts, which is key to the CCRWQCB's decision on the WDRs. Moreover, the list of proposed disposal areas in the WDRs omits identification of any discharge location consistent with County condition of approval No. 97, which requires at least 10 % of the treated wastewater to be reserved for environmental enhancement, including the protection of local Environmentally Sensitive Habitat Areas.

Knowledge of the specific wastewater discharge locations is of the outmost importance, especially because recycled wastewater is not contaminant free. (See, e.g., Comments of Edo McGowan, attached as exhibit 1.) It contains antibiotic resistant genes (ARGs) and contaminants of emerging concern (CECs). CECs include persistent organic pollutants (e.g., chemicals used in flame retardants; pharmaceuticals and personal care products; veterinary medicines; endocrine-disrupting chemicals (e.g. synthetic estrogens and androgens affecting hormonal functions in aquatic organisms); and nanomaterials (e.g., carbon nanotubes). (See U.S. E.P.A. OW/ORD Emerging Contaminants Workgroup, *Aquatic Life Criteria for Contaminants of Emerging Concern* (June 3, 2008), attached hereto as exhibit 2, at 2.) Numerous scientific articles and studies have investigated both CECs and ARGs in treated wastewater and have documented the health risks they pose to humans and wildlife -- addressing, for example, limb malformations and behavioral abnormalities, decreased reproduction, increased susceptibility to predation, and increased vulnerability to parasites, disease and UV radiation in amphibians and fish. (See *id.*; Petrovic, et al., *Analysis and Removal of Emerging Contaminants in Wastewater and Drinking Water* (2003), attached hereto as exhibit 3, at 3-5; Pruden, et al., *Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado* (2006) 40 Envtl. Science & Technology 7445, attached hereto as exhibit 4, at 1.)

The Porter-Cologne Water Quality Control Act requires identification of all discharge locations before the WDRs may be approved. By Water Code section 13263, the CCRWQCB must "prescribe requirements as to the nature of any proposed discharge . . ., *with relation to the*

(*Id*.) Evidently, 25 unspecified locations for agricultural reuse irrigation plus ten unspecified locations for urban reuse irrigation amounts to more than four discharge points. The staff report and the WDRs also reference as an attachment a barely legible map which shows several tiny squares scattered throughout an approximately six-square mile area, vaguely suggesting some of the reuse locations, all without indicating what agricultural lands or urban properties are being referenced, what they are being used for (e.g., turf at local schools, presenting risks of human contact with wastewater?), or whether their needs for recycled water are actually unmet. The map is vaguely illustrative and nonbinding at best. It does not refer to any specific selected wastewater discharge locations. That much is clear from the staff report.

conditions existing in the disposal area or receiving waters upon, or into which, the discharge is made or proposed." (Water Code, § 13263, subd. (a), emphasis added.) The plain language of this statutory provision makes clear that for the CCRWQCB to be able to prescribe WDRs as to the nature of any discharge proposed by the County, disclosure and analysis of site-specific baseline conditions at the discharge locations is necessary. The Legislature did not intend for regional water quality control boards to prescribe requirements "*with relation to* conditions existing in the disposal area" (Water Code, § 13263, emphasis added), without informing themselves, disclosing and evaluating those conditions.

The conditions existing in the disposal area or receiving waters impacted by proposed discharges are the physical baseline conditions affected by a project subject to WDRs. Therefore, CEQA, too, requires that the CCRWQCB identify the wastewater discharge locations. (See *Communities for a Better Environment v. South Coast Air Quality Management Dist.* (2010) 48 Cal.4th 310, 321 ["the impacts of a proposed project are ordinarily to be compared to the actual environmental conditions existing at the time of CEQA analysis, rather than to allowable conditions defined by a plan or regulatory framework"; thus, "the baseline for CEQA analysis must be the 'existing physical conditions in the affected area' [citation], that is, the ' "real conditions on the ground" ' [citations]"]; *County of Amador*, 76 Cal.App.4th 931, 952 ["It is only against [the] baseline [of the existing environmental conditions] that any significant environmental effects can be determined"]; *Sundstrom v. County of Mendocino* (1988) 202 Cal.App.3d 296, 308-309.)

In *Sundstrom*, the project applicant wanted to build a motel which necessitated the construction of a private sewage treatment plant. The mitigated negative declaration for the project included a requirement that the applicant obtain approval of a sludge disposal plan from the regional water quality control board, but did not disclose the sludge disposal location. The appellate court held that by dismissing the impact as insignificant without disclosure of a suitable sludge disposal site, the permitting agency failed to comply with CEQA.

A related CEQA issue pertaining to the LOWRF project's recycled water program arises out of the proposed condition that the County "develop an Engineering Report on the Production, Distribution and Use of Recycled Water" for later review and approval by the CCRWQCB's Executive Director. (WDRs at 13; CCRWQCB staff report at 4.) But environmental review and approval of the production, distribution and use of recycled water -- an integral element of the LOWRF project -- cannot be so segregated from the project, deferred to the future and delegated away by the permitting agency's decisionmaking body. As *Sundstrom* explains, at page 307:

> "A study conducted after approval of a project will inevitably have a diminished influence on decisionmaking. Even if the study is subject to administrative approval, it is analogous to the sort of post hoc rationalization of agency actions that has been repeatedly condemned in decisions construing CEQA."

Should the CCRWQCB not wish to conduct SEIR review, which would adequately inform its decision on the WDRs, it has the option of denying the WDRs outright. (See § 21080, subd. (b)(5) [an agency's rejection of a project is exempt from CEQA review].) As a responsible agency under CEQA, the CCRWQCB may indeed "refuse to approve" the WDRs "to avoid direct or indirect environmental effects of that part of the project which [as the] responsible agency [it is] called on to

carry out or approve." (Guidelines, § 15042.) However, if the CCRWQCB does not choose such course of action, the environmental issues raised above and those identified in our addendum (and supporting attachments) must be addressed and resolved in an SEIR. An SEIR should consider in-depth reasonable alternatives, including a septic tank effluent pumping system or other alternatives and mitigation measures that more effectively avoid or minimize the environmental impacts of the LOWRF project as currently proposed, while demanding substantially less public funding (and hence less economic burden on the community that must pay for it).

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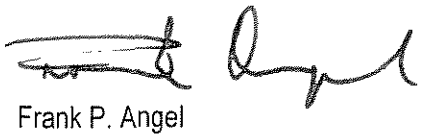

Frank P. Angel

Enclosures

cc: Harvey Packard, Section Manager (hpackard@waterboards.ca.gov)

Exhibit 1

3/1/11 Bd Mtg. Item 5
Los Osos
Deadline: 2/22/11 by 12 noon

commentletters - 3/1/2011 BOARD MEETING

From: Edo McGowan <edo_mcgowan@hotmail.com>
To: <commentletters@waterboards.ca.gov>, <mkeeling@waterboards.ca.gov>, <edo...
Date: Monday, February 21, 2011 2:16 PM
Subject: 3/1/2011 BOARD MEETING
Attachments: antibiotic resistance.jpg; antibiotic resistance-SB.jpg

To: SWRCB & SLO RWQCB, Comments on Item #5 **3/1/2011 BOARD MEETING**

Re: Clerk to the Board at commentletters@waterboards.ca.gov. "**3/1/2011 BOARD MEETING**

DIVISION OF FINANCIAL ASSISTANCE

Item #5

Consideration of a proposed Resolution authorizing funding from the Clean Water State Revolving Fund (CWSRF) to the County of San Luis Obispo Los Osos Wastewater Project (Project), CWSRF Project No. C-06-5230.

The Project will offset potable water use in the Los Osos Groundwater Basin (Groundwater Basin) with California Title 22 disinfected, tertiary-treated recycled water. Recycled water will be **used for urban** and **agricultural irrigation** at sites that currently rely on potable water.

Comment: 1)The moneys allocated to this project should include testing for antibiotic resistant genes (ARGs) in the finished disinfected Title 22 recycled water. If this can not be done because the water is as yet unavailable, a surrogate test could be conducted on similar systems producing this water around the state for ARGs. Sewer plants that are proposed for preparation of recycled water must be capable of also removing CECs and ARGs from the finished product. This is currently not often the situation, thus: 3) Title 22 recycled water as typically produced represents a potentially cumulative and unnecessary adverse impact on human and environmental health.

This comment is is intended to be detailed so that the critical information information is available to the State and Regional Boards and their respective staff, thus allowing for a thoughtful development of policy and regulatory controls on the subject project. Antibiotic resistance is now a fact of life and thus it will be important for the boards and their staff to have some acquaintance with the subject. This is especially critical because the currently designed sewer plants generate the production of antibiotic resistant organisms which are then discharged in large numbers into the environment.

Fm: Dr Edo McGowan, Medical Geo-hydrology, Medical Hydropathology Working Group

The subject of antibiotic resistance, as discussed herein, is important for several reasons. Both human and veterinary medicine are expending considerable resources nationally in attempting to control the accelerating rates of antibiotic resistance. At the same time, resistance is rapidly destroying the remaining antimicrobial drugs that currently exist. To add insult to injury, the pharmaceutical industry seems disinterested in investing in new antimicrobials. Thus the tools available to medicine are diminishing. Concomitantly, the sewer plants, as currently designed and operated are releasing large volumes of antibiotic resistant microbes into the environment, thereby sabotaging the efforts of both human and veterinary medicine to control antibiotic resistance and consequently sewer plants and their byproducts are endangering public health and herd health.

That sewer plants as currently designed and operated do generate and discharge antibiotic resistant bacteria is beyond dispute. The Wastewater Research Division, Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio, conducted a protracted study on the ability of currently designed sewer plants to generate antibiotic resistant bacteria, which are then discharged. This US/EPA study was conducted in the late 1970s and reported in the early 1980s. The report based on the US/EPA study (1982) cited former studies reaching back another decade into the past and these cited studies said the same thing. Thus, although the information could hardly be considered as new, i.e., it has been around for nearly 4-decades, it seems not to have worked itself into the regulatory processes that control sewer plants. Since the State is **prime** for purposes of the Clean Water Act, it is time for the State and its Regional Boards to recognize this shortcoming, hence the need for the detail in this comment. Here is some extracted text from the report of the study conducted by the US/EPA:
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC241834/pdf/aem00183-0119.pdf

"Several researchers have pointed out that wastewater, treated or untreated, is a primary contributor of bacteria to the aquatic ecosystem (12, 16, 17, 20, 27, 29). Studies have been conducted which demonstrate that significant numbers of multiple drug-resistant coliforms occur in rivers (17), bays (9), bathing beaches (28), and coastal canals (13). Waters contaminated by bacteria capable of transferring drug resistance are of great concern since there is the potential

for transfer of antibiotic resistance to a pathogenic species.

When bacteria which carry transmissible R-factors (R+ bacteria) are ingested by a human host, the R-factors may transfer into commonly occurring bacteria of the gastrointestinal tract (32). These organisms may subsequently transfer this resistance to pathogenic organisms, resulting in reduced efficacy of antimicrobial chemotherapy in the event of an infection. In vivo studies have shown that when individuals carrying R+ bacteria are subjected to antibiotic therapy, py, these organisms flourish and transfer their resistance to other bacteria (25)."

12. Geldreich, E. E. 1972. Water-borne pathogens, p. 207-241. In R. Mitchell (ed.), Water pollution microbiology. Wiley-Interscience, New York.
16. Grabow, W. O. K., O. W. Prozesky, and L. S. Smith. 1974. Drug-resistant coliforms call for a review of water quality standards. Water Res. 8:1-9.
17. Grabow, W. O. K., M. VanZyl, and O. W. Prozesky. 1976. Behavior in conventional sewage purification processes of coliform bacteria with transferable or non-transferable drug resistance. Water Res. 10:717-723.
20. Linton, K. B., M. H. Richmond, R. Bevau, and W. A. Gillespie. 1974. Antibiotic resistance and R-factors in coliform bacilli isolated from hospital and domestic sewage. J. Med. Microbiol. 7:91-103.
27. Smith, H. W. 1970. Incidence in river water of Escherichia coli containing R-factors. Nature (London) 228:1286-1288.
29. Sturtevant, A. B., Jr. and T. W. Feary. 1969. Incidence of infectious drug resistance among lactose-fermenting bacteria isolated from raw and treated sewage. Appl. Microbiol. 18:918-924.

Project (No. C-06-5230)

The project (No. C-06-5230) discusses both municipal irrigation with recycled water and also the use of that water on agricultural lands. This comment will first discuss the use in agriculture. Following that will be a discussion on the use in municipal irrigation and accompanying human health issues. Additional attention should be accorded to the area of effluent discharge, i.e., the location and length of the outfall, the currents and tides and thus the ability for the outfalled sewage effluent to contaminate coastal areas and beach sands. To illustrate this effect, a separate note discussing the Montecito Sanitary District's outfall is attached at the end of this comment.

AGRICULTURE

It is necessary to discuss how irrigation with Title 22 recycled water may impact both the food produced, hence human health and also impact the underlying agricultural base that produces such food. It is well established that the food borne disease outbreaks in the Salinas Valley, through media attention, had badly crippled the area's income, even for farms not even connected with the problem. Nonetheless, the "problem" became a widespread economic disaster.

It is also well established that current water quality tests used by the State of California fail to reflect the actual presence of pathogens often found in the finished and disinfected Title 22 recycled water. This fact is amply demonstrated by the 2004 study by WERF (00-PUM-2T). The essence of that report's findings may be summed by the following sentence: "The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed."

This failure is also reflected by my work with finished disinfected Title 22 recycled water tested at two separate sewer districts, in which water we found multi-antibiotic resistant bacteria, see attachments which are photographs of disc diffusion tests of the recycled water showing the extent of the resistance. If you are unfamiliar with reading these test results, please do not hesitate to contact me for assistance. In the case of the finished Title 22 recycled water produced by the El Estero plant, the bacteria were resistant to 11 of the 12 challenge antibiotics. Water from the Goleta plant demonstrated resistance to 4 of the 12 test antibiotics. Unless there are some uncharacteristic factors related to these two plants that produce recycled water per state criteria that make them different from all other plants within the state, we should also find these results generally in most other plants that use these same criteria.

When this water is used to irrigate crops consumed raw, the bacteria and their genetic fragments can, through the consumption of the produce, be transferred to the human gut biota. These resistant bacteria and their genes are able to thus impact both the internal commensal (gut) biota and immune system.

Sjolund (2005) has found that once in the gut, the genetic material has a very long residence life, years. Additionally, once ingested, the plasmids may be transferred to and through normal flora into pathogenic bacteria found in humans or animals, making later treatment with particular antibiotics ineffective (US/EPA also noted this transfer). Additionally, one must consider that the transfer of genetic information from these organisms to more robust organisms might contribute to increased resistance in higher-grade pathogens by interspecies transfer. Sjolund et al go on to note that since populations of the normal biota are large, this affords the chance for multiple and different resistant variants to develop. This accordingly enhances the risk for spread to populations of pathogens. Consequently, by the use of sewage generated recycled water, as currently produced, there is a revolving door between the human population and the sewer plant, especially when foodstuffs are locally grown and consumed when raised on recycled water that is improperly treated.
What goes on food crops which are consumed raw can have long lasting effects. When humans defecate there is production of a fecal veneer around the anal skin. Thus the fecal veneer forming on the perineum would see colonization of adjoining orifices. There are recent studies on vaginal flora were females who had no contact with antibiotics were found with vaginal flora containing drug resistant bacteria. For the pregnant female, this may place the pregnancy at risk, especially if *Listeria monocytogenes* is found in water and soil. According to the Center of Disease Control (CDC), an estimated 2,500 persons become seriously ill each year with *Listeria monocytogenes* in the United States and among these, 500 will die. According to research, pregnant women account for 27% of these cases. CDC claims that pregnant women are 20 times more likely to become infected than non-pregnant healthy adults.
Vegetables can become contaminated from the soil, especially of the bacteria are brought in with the irrigation water. Such an inner connected series as irrigation with recycled water, contamination of crop, consumption of crop, transfer of resistance to gut, movement to fecal veneer, and establishment of resistant contamination in vaginal flora as described immediately above would provide but a single example for project No. C-06-5230, without adequate controls, to become a public health risk.

It is important to understand that there are two basic types of antibiotics with which medicine (human or veterinary) work: bacteriostatic and bacteriocidal. A good primer on this difference has been produced by the National Foundation for Infectious Disease and may be found at http://www.nfid.org/pdf/id_archive/antibiotictherapy.pdf.

Examples:

Bactericidal Drugs: Streptomycin, Sulfonamides, Aminoglycosides
Bacteriostatic Drugs: Tetracycline, Penicillin, Chloramphenicol

Many antibiotics are bacteriostatic and thus merely arrest the growth of bacteria but do not kill them, that job is left for the immune system. But if the immune system is also disrupted by, for example diabetes, and then hit with a chlorine-resistant bacteria, the job of quelling infections, hence the job of medicine, becomes far more complicated. The immune system is designed to produce a chlorine-like material used by the white blood cells to kill bacteria. But if these bacteria are chlorine resistant, this greatly challenges the immune system. If that immune system is already compromised: the very young, the very old, the diabetic, those on immune supressors, etc., the job of medicine is much increased.

Perhaps the case of methicillin resistant *Staphylococcus aureus* (MRSA) is an apt example. MRSA, out of all the various drug resistant pathogens, by itself, now kills more Americans than AIDS, this according to the CDC. Matt Wook Chang (see abstract below), in studying the response of bacteria to chlorine is finding that chlorine up-shifts the capacity of pathogens to cause disease by causing induction of major *virulence* genes. In working with methicillin resistant *Staphylococcus aureus* (MRSA), Chang finds that virulence genes are up-regulated by exposure to chlorine. This response is not limited to this one pathogen but is widely seen amongst bacteria. Dr Marlyn Roberts is finding MRSA along beaches and this issue of beach sands will be discussed later in this document. It is important to note, however, that beach sands offer an excellent medium for the growth and sustenance of pathogenic bacteria.

Thus although pathogens that might otherwise seldom meet in nature to exchange genetic information, upon entering sewer plants are finding themselves forced into close contact within these sewer plants with high levels of gene exchange and then exposed to chlorine. In this situation, the opportunities for development of both enhanced resistance and virulence are elevated. Because bacteria under stress can also enter a dormant state that is not visible to the standard tests, they are missed in the single indicator tests typically used in testing recycled water. This dormant state is called viable but non-culturable (VBNC). WERF has published on this dormant state and has shown that the standard tests used in wastewater and for wastewater byproducts may substantially under represent actual bacterial numbers **by several magnitudes**. Thus, in these conditions, which are common, the standard test results are giving false negatives and thus failing to serve their role in public health protection. We have noted this VBNC when we test finished Title 22 recycled water with the state approved MPN test. When we tested finished disinfected Title 22 recycled water just as it leaves the plant and again at the point of use we got dramatically different results. If we test just as the water is leaving the plant, we get non-detect or very low numbers with this standard testing procedure. When, however, we test this same water with the same test procedure at the point of use the numbers are often quite high and at times completely off the chart. We feel that this dramatic difference is related to resuscitation of bacteria in the VBNC state. It could also reflect the shedding of biofilms. Testing would help differentiate these two potentials. Personally, I think it is both and once the delivery system has an established biofilm that is shedding, the issue of public health is exacerbated. In using the disc diffusion tests as compared to the single indicator MPN test at both point of release and point of use, we get multi-antibiotic resistant bacteria. Thus the standard single indicator tests are not reflecting reality.

Some sewer plants producing recycled water are also using UV light as a disinfectant. There are flaws with this process that warrant some discussion. As noted in the 1982 report from the US/EPA study that demonstrated generation of antibiotic resistance by sewer plants, it will be noticed that UV not only enhanced antibiotic resistance but failed to kill up to 40% of certain

target bacteria. More recently in discussion with Dr. Amy Pruden who works with ARGs, it was mentioned that in close examination of UV disinfection results, although the bacteria were killed, the critical DNA that conferred resistance was not affected. If one were to review the 1928 work of Dr Fred Griffith, it will be found that live bacteria can extract genetic information from heat-killed bacteria.Thus merely killing bacteria with UV is insufficient for public health purposes.

This observation, the non destruction of DNA by UV, by Dr. Pruden is a significant finding because there is a strong reliance on UV in some systems. Dr. Pruden also notes that ARGs are unaffected by chlorine at levels typically used in water quality control and are so small that they will pass through typical filtering systems used by water treatment plants. Consequently, there is the opportunity to see ARGs and gene fragments, which are not amenable to the standard tests, making it through treatment works into the environment. Dr. Pruden is set up to test water samples for ARGs and it is suggested here that the State and thus its Regional Boards contact Dr Pruden to discuss testing of recycled water for ARGs. She may be reached through her university number at (540) 231-3980.

Antibiotic resistant genes that are common to reclaimed water are now also found in drinking water. The fact that sewer plants are generators of antibiotic resistant pathogens whose genes are generally easily taken up and multiplied by the gut biota is an area needing recognition by both the Stare and regional Boards and their respective staff. The ARGs, as I have previously noted, are so small that they pass through many types of filters normally used in water treatment and are not affected by chlorine levels typically used in water treatment. This fact then needs to be inculcated with standards and conditions for permitted uses. For example, if one were to examine the files at the SLO office for the plant in Monterey producing recycled water for the Salinas area, it might be noted that the filters (at least one) was being pushed at 150% of its rated capacity. This over-charge of the filter may well see ARGs moving through the system in large numbers. But based on current standardized tests, that would never be noted, even though the issue of ARGs and health are not disconnected.

The current standards also use proxy or surrogate tests to ascertain water quality. These proxy tests offer a template approach where the template is overlain on the system and if there is a fit, it is assumed that the water is safe. That template related to water clarity and coliform numbers is flawed. The Pomona virus study and its analysis discuss some of these surrogate tests related to water clarity and coliform numbers. In its discussion of this the State itself notes that there is no scientific basis for the reliance on this proxy test. Nonetheless, the regulatory community continues to allow the use of these tests. Much new scientific information has been brought forward since the Pomona study. Additionally, the stakes are now much higher because we are not only losing the battle with antibiotic resistant organisms, but the standards do not consider ARGs, thus there is a serious need to reappraise the tests which underlay the standards.

When we discuss the use of recycled water for irrigation of crops, it is important to understand that crops can internalize bacteria and thus no amount of washing at the kitchen sink has effect on such internalized bacteria. This internalization of bacteria was demonstrated in the 2002 Rutgers University study (see abstract below).

In the case of resistant bacteria and their genes, the bacteria can also interact with the soil commensal biota thus their containment within the soil in which the crops are grown becomes a reservoir for pathogens. This allows for a standing body of bacteria that can enter the roots, thus transferring to the edible portions. As shown by Chad Kinney (see abstract below) certain pharmaceuticals can accumulate in soils and these pharmaceuticals have been shown to be brought in with the recycled (reclaimed) water. The accumulation allows for two detractors, one is the eventual buildup of antibiotics like erythromycin to levels that may key off the development or maintenance of resistance and also, as CECs, may themselves be taken into the crop.

As currently designed and thus operated, many sewer plants can not effectively remove materials in solution. That this is a well documented fact may be seen by the pharmaceuticals now found in the water resources of this nation. There are ways around this with different available technology. That technology can be discussed in a different venue and I am pleased to do so.

A lot of food is now being irrigated with reclaimed sewer water. The State of California allows reclaimed water to be used on crops consumed raw. If this water were properly treated, that would not be a problem. Unfortunately, under current standards and the way sewer plants are designed and run, it can not be properly treated. Thus, leafy greens coming out of ag production areas may be highly suspect, this includes the certified organic. The abstracts below will give you some idea of this issue. Thus while the water may meet standards, it is by no means necessarily safe.

There are provisions in law which allow the Regional Boards to develop standards in areas not covered by current standards. Thus the discussions within this comment document are intended to provide an impetus toward the setting of corrective standards by the boards themselves where the CDPH has not entered the area and where there is a crying need for such standards.

My group is running tests on finished disinfected Title 22 recycled water and finding multi-antibiotic resistant bacteria in the finished product that goes on crops consumed raw. Additionally, as noted above, crops can internalize bacteria and thus no amount of washing at the kitchen sink has effect. Additionally, the plants can internalize pollutants, some of which will biomagnify, i.e., become concentrated within the plants. For example, if a field has too much organochlorine pesticide, one need only plant carrots to clean the soil. The carrots extract the pesticide and bioaccumulate it within the plant's tissues. This is the process called phytoremediation and it is a highly developed and scientifically validated process used globally. We did some of the initial work on developing this process when I was a grad student in the early 1970s at the Environmental Toxicology Department at UC Davis. We tested this with dieldrin and carrots.

Typical sewer plants do not do well with materials in solution and even RO has considerable difficulty in removing flame retardants and about 5% of these materials are found to move through RO. Additionally, certain antibiotics are also not removed by RO and there are recorded failure rates of 20%—this also assumes that these membranes are kept in pristine condition.

Interestingly, the systems that use reclaimed water for crop irrigation do not even go the the extent of using RO.

Chad Kinney, et al (2005) has demonstrated that soil irrigated with recycled water will accumulate pharmaceuticals. These then may biomagnify within the crop grown in that soil.

As to the biomagnification of endocrine disrupters in crops, this is again amply demonstrated in the literature. We do know that endocrine disrupters found in discharged wastewater can, and have, impacted several animal species, birds, fish, amphibians, and worms. Thus, there is potential but unknown impact on humans, especially as such would relate to fetal and first trimester development. Consequently by irrigating crops with recycled water with entrained endocrine mimics and antibiotic resistant microbes, including their genes, the public is being essentially put into a large unauthorized and potentially unethical human experiment.

The levels of endocrine mimics found in recycled water used to irrigate crops must be considered. Bacteria are not the only thing that may be taken into the plants and moved into the edible portions.

IRRIGATION OF MUNICIPAL GREENSCAPE

When this water is used to irrigate greenscape, many of the above discussed problems arise. The bacteria and their genetic fragments can, through the contact, reach the mouth and then enter the human gut biota, moving therefrom to body orifices. As we have seen above, these resistant bacteria and their genes are able to thus impact both the internal commensal (gut) biota and immune system.

Rusin and Gerba have published on finger to mouth transfer of pathogens, see abstracts below. With sprinkler irrigation of recycled water, the aerial transport to off-site surfaces happens and these contaminated surfaces (fomites) allow the transfer of pathogens to humans. There is a good body of literature on sprinkler generation of aerosols that can carry pathogens for considerable distances. The German government has established a set back of 300 meters between sprinklers and habitation. This is not now the case in California where sprinkler irrigation is just across the street from residential neighborhoods. Basum, see below, noted significant drift distances with sprinkler irrigation. This then becomes critical for irrigation around schools and thus on school playing fields where children with immature immune systems are present and have contact with fomites. I think it is not pushing the point to consider the transfer of resistant organisms to school children and then the liability issues that might arise from and to school districts for contamination.

CITATIONS

Sjolund et al. (2005) Emerging Infectious Diseases (Vol. 11, # 9, Sept 2005 @ p. 1389 et seq)

Sara Firl. **The Importance of Municipal Sewage Treatment in the Spread of Antibiotic Resistance**
106th General Meeting of the American Society for Microbiology
May 21-25, 2006, Orlando, Florida. (Session 041/Q, Paper Q-032)

Our study determined that substantial numbers of antibiotic-resistant bacteria were present in municipal wastewater, and that the existing treatment infrastructure did not adequately prevent release of antibiotic-resistant bacteria into the environment.

Validity of the Indicator Organism Paradigm for Pathogen Reduction in Reclaimed Water and Public Health Protection†

Valerie J. Harwood,[1]* Audrey D. Levine,[2] Troy M. Scott,[3] Vasanta Chivukula,[1] Jerzy Lukasik,[3] Samuel R. Farrah,[4] and Joan B. Rose[5]

The validity of using indicator organisms (total and fecal coliforms, enterococci, *Clostridium perfringens*, and F-specific coliphages) to predict the presence or absence of pathogens (infectious enteric viruses, *Cryptosporidium*, and *Giardia*) was tested at six wastewater reclamation facilities. Multiple samplings conducted at each facility over a 1-year period. Larger sample volumes for indicators (0.2 to 0.4 liters) and pathogens (30 to 100 liters) resulted in more sensitive detection limits than are typical of routine monitoring. Microorganisms were detected in disinfected effluent samples at the following frequencies: total coliforms, 63%; fecal coliforms, 27%; enterococci, 27%; *C. perfringens*, 61%; F-specific coliphages, ~40%; and enteric viruses, 31%. *Cryptosporidium* oocysts and *Giardia* cysts were detected in 70% and 80%, respectively, of reclaimed water samples. Viable *Cryptosporidium*, based on cell culture infectivity assays, was detected in 20% of the reclaimed water samples. No strong correlation was found for any indicator-pathogen combination. When data for all indicators were tested using discriminant analysis, the presence/absence patterns for *Giardia* cysts, *Cryptosporidium* oocysts, infectious *Cryptosporidium*, and infectious enteric viruses were predicted for over 71% of disinfected effluents. The failure of measurements of single indicator organism to correlate with pathogens suggests that public health is not adequately protected by simple monitoring schemes based on detection of a single indicator, particularly at the detection limits routinely employed. Monitoring a suite of indicator organisms in reclaimed effluent is more likely to be predictive of the presence of certain pathogens, and a need for additional pathogen monitoring in reclaimed water in order to protect public health is suggested by this study. (Applied and Environmental Microbiology, June 2005, p. 3163-3170, Vol. 71, No. 6)

Lettuce Plants Internalize Bacteria

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Lettuce that has been fertiziled with manure or irrigated with water that is contaminated with *E. coli* O157:H7 can take the bacteria up through its root system and internalize it inside its leaves, resisting traditional external sanitizing methods. Researchers from Rutgers University report their findings in the January 2002 issue of the journal *Applied and Environmental Microbiology*. "In recent years, *E. coli* O157:H7 has been isolated with increasing frequency from fresh produce, including bean sprouts, cantloupes, apples and leaf lettuce. The mechanisms by which the pathogen is introduced into the lettuce plant are not fully understood," say the researchers.

The researchers tested the hypotheses that the source of the contamination may be poorly treated manure (it is estimated that the pathogen is present in over 8 percent of dairy and beef cattle) or irrigation water that has been contaminated with cattle feces. The bacteria were isolated from plants grown using either medium, but interestingly, the researchers found bacteria in the inner tissues of the plants.

"We have demonstrated that lettuce grown in soil containing contaminated manure or irrigated with contaminated water results in contamination of the edible portion of the lettuce plant," say the researchers. "Moreover, the results suggest that edible portions of a plant can become contaminated without direct exposure to a pathogen but rather through transport of the pathogen into the plant by the root system. The inaccessibility of a large number of organisms, as a consequence of their subsurface location, is perhaps the reason for the lack of effectiveness of surface-sanitizing treatments." **(E.B. Solomon, S. Yaron, K.R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Applied and Environmental Microbiology, 68: 397-400.)**

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Wastewater Derived Pharmaceuticals In Soil Irrigated With Reclaimed Water

Environmental Toxicology and Chemistry

Article: pp. 317–326 | Abstract | PDF (143K)

PRESENCE AND DISTRIBUTION OF WASTEWATER-DERIVED PHARMACEUTICALS IN SOIL IRRIGATED WITH RECLAIMED WATER

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Three sites in the Front Range of Colorado, USA, were monitored from May through September 2003 to assess the presence and distribution of pharmaceuticals in soil irrigated with reclaimed water derived from urban wastewater. Soil cores were collected monthly, and 19 pharmaceuticals, all of which were detected during the present study, were measured in 5-cm increments of the 30-cm cores. Samples of reclaimed water were analyzed three times during the study to assess the input of pharmaceuticals. Samples collected before the onset of irrigation in 2003 contained numerous pharmaceuticals, likely resulting from the previous year's irrigation. Several of the selected pharmaceuticals increased in total soil concentration at one or more of the sites. The four most commonly detected pharmaceuticals were erythromycin, carbamazepine, fluoxetine, and diphenhydramine. Typical concentrations of the individual pharmaceuticals observed were low (0.02–15 µg/kg dry soil). The existence of subsurface maximum concentrations and detectable concentrations at the lowest sampled soil depth might indicate interactions of soil components with pharmaceuticals during leaching through the vadose zone. Nevertheless, the present study demonstrates that reclaimed-water irrigation results in soil pharmaceutical concentrations that vary through the irrigation season and that some compounds persist for months after irrigation.

Keywords: Pharmaceuticals, Wastewater, Reclaimed water, Soil, Leaching tendencies

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Viability of a low-pressure nanofilter in treating recycled water for water reuse applications: A pilot-scale study

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Abstract

The purpose of this study was to investigate the potential of a low-pressure nanofiltration (NF) membrane for treating recycled water for indirect potable water reuse applications. In particular, the tradeoffs in choosing low-pressure NF over reverse osmosis (RO) were investigated including whether or not significantly lowering operating pressures/costs would result in diminished permeate water quality. A NF membrane (Dow/Filmtec NF-4040) with high permeate productivity was selected for pilot-scale testing over a period of 1200 h at a water reuse facility employing conventional RO membranes for treating tertiary treated wastewater effluent prior to aquifer recharge. The novel application of an NF membrane in treating wastewater effluent for water reuse applications permitted a comprehensive screening of NF permeate water quality and allowed for the investigation of trace organic contaminant rejection on pilot scale with environmentally relevant feed water concentrations. Results from pilot-scale testing highlighted the selectivity of NF membranes in removing organic solutes present in wastewater effluents at the parts-per-trillion level. While operating pressures were by a factor of 2–3 lower than conventional RO membranes, and bulk and trace organic rejection generally exceeded 90 percent, not surprisingly, the rejection of monovalent ions such as nitrate was poor. The poor-to-moderate rejection of monovalent ions, however, resulted in lowered brine stream total dissolved solids concentration and sodium adsorption ratio as compared with the brine stream of conventional RO membranes, which may be beneficial for brine disposal strategies.

Toxicogenomic response to chlorination includes induction of major virulence genes in Staphylococcus aureus

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Abstract

Despite the widespread use of chlorination for microbial control in aqueous environments, cellular response mechanisms of human pathogens, such as Staphylococcus aureus, against chlorination remain unknown. In this work, genome-wide transcriptional analysis was performed to elucidate cellular response of S. aureusto hypochlorous acid, an active antimicrobial product of chlorination in aqueous solution. Our results suggest that hypochlorous acid repressed transcription of genes involved in cell wall synthesis, membrane transport, protein synthesis, and primary metabolism, while amino acid synthesis genes were induced. Furthermore, hypochlorous acid induced transcription of genes encoding major virulence factors of S. aureus, such as exotoxins, hemolysins, leukocidins, coagulases, and surface adhesion proteins, which all play essential roles in staphylococcal virulence. This work implies that chlorination may stimulate production of virulence factors, which provides new insight into host-pathogen interactions and effects of chlorine application for microbial control.

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J Appl Microbiol. 2002;93(4):585-92.

Comparative surface-to-hand and fingertip-to-mouth transfer efficiency of gram-positive bacteria, gram-negative bacteria, and phage.

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Abstract

AIMS: To determine the transfer efficiency of micro-organisms from fomites to hands and the subsequent transfer from the fingertip to the lip.
METHODS AND RESULTS: Volunteers hands were sampled after the normal usage of fomites seeded with a pooled culture of a Gram-positive bacterium (Micrococcus luteus), a Gram-negative bacterium (Serratia rubidea) and phage PRD-1 (Period A). Activities included wringing out a dishcloth/sponge, turning on/off a kitchen faucet, cutting up a carrot, making hamburger patties, holding a phone receiver, and removing laundry from the washing machine. Transfer efficiencies were 38.47% to 65.80% and 27.59% to 40.03% for the phone receiver and faucet, respectively. Transfer efficiencies from porous fomites were <0.01%. In most cases, M.luteus was transferred most efficiently, followed by phage PRD-1 and S. rubidea. When the volunteers' fingertips were inoculated with the pooled organisms and held to the lip area (Period B), transfer rates of 40.99%, 33.97%, and 33.90% occurred with M. luteus, S. rubidea, and PRD-1, respectively.
CONCLUSIONS: The highest bacteral transfer rates from fomites to the hands were seen with the hard, non-porous surfaces. Even with low transfer rates, the numbers of bacteria transferred to the hands were still high (up to 10(6) cells). Transfer of bacteria from the fingertip to the lip is similar to that observed from hard surfaces to hands.
SIGNIFICANCE AND IMPACT OF THE STUDY: Infectious doses of pathogens may be transferred to the mouth after handling an everyday contaminated household object.

Significance of Fomites in the Spread of Respiratory and Enteric Viral Disease▽

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Worldwide annually there are 1.7 million deaths from diarrheal diseases and 1.5 million deaths from respiratory infections (56). Viruses cause an estimated 60% of human infections, and most common illnesses are produced by respiratory and enteric viruses (7, 49). Unlike bacterial disease, viral illness cannot be resolved with the use of antibiotics. Prevention and management of viral disease heavily relies upon vaccines and antiviral medications (49). Both vaccines and antiviral medications are only 60% effective (39, 49). Additionally, to date there are no vaccines or antiviral drugs for most common enteric and respiratory viruses with the exception of influenza virus and hepatitis A virus (HAV). Consequently, viral disease spread is most effectively deterred by preclusion of viral infection.

Increases in population growth and mobility have enhanced pathogen transmission and intensified the difficulty of interrupting disease spread (14). Control of viral disease spread requires a clear understanding of how viruses are transmitted in the environment (27). For centuries it was assumed that infectious diseases were spread primarily by the airborne route or through direct patient contact, and the surrounding environment played little or no role in disease transmission (19, 27). Up until 1987 the Centers for Disease Control and the American Hospital Association focused on patient diagnosis due to the belief that nosocomial infections were not related to microbial contamination of surfaces (19). Over the years studies have changed the perspective on viral transmission to include a more complex multifactorial model of disease spread (27). There is now growing evidence that contaminated fomites or surfaces play a key role in the spread of viral infections (3, 7, 38, 71).

Viral transmission is dependent on interaction with the host as well as interaction with the environment (60). Viruses are probably the most common cause of infectious disease acquired indoors (7, 71). The rapid spread of viral disease in crowded indoor establishments, including schools, day care facilities, nursing homes, business offices, and hospitals, consistently facilitates disease morbidity and mortality (71). Yet, fundamental knowledge concerning the role of surfaces and objects in viral disease transmission is lacking, and further investigation is needed (52, 60, 61). The goal of this article was to use existing published literature to assess the significance of fomites in the transmission of viral disease by clarifying the role of fomites in the spread of common pathogenic respiratory and enteric viruses.

Comparison of Coliphage and Bacterial Aerosols at a Wastewater Spray Irrigation Site

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Microbiological aerosols were measured on a spray irrigation site at Fort Huachuca, Ariz. Indigenous bacteria and tracer bacteriophage were sampled from sprays of chlorinated and unchlorinated secondary-treatment wastewaters during day and night periods. Aerosol dispersal and downwind migration were determined. Bacterial and coliphage f2 aerosols were sampled by using Andersen viable type stacked-sieve and high-volume electrostatic precipitator samplers.

Bacterial standard plate counts averaged 2.4 x 105 colony-forming units per ml in unchlorinated effluents. Bacterial aerosols reached 500 bacteria per m3 at 152 m downwind and 10,500 bacteria per m3 at 46 m. Seeded coliphage f2 averaged 4.0 x lo, plaque-forming units per ml in the effluent and were detected 563 m downwind. Downwind microbial aerosol levels were somewhat enhanced by nighttime conditions. The median aerodynamic particle size of the microbial aerosols was approximately 5.0 p.m. Chlorination reduced wastewater bacterial levels 99.97% and reduced aerosol concentrations to near background levels; coliphage f2 was reduced only 95.4% in the chlorinated effluent and was readily measured 137 m downwind. Microbiological source strength and meteorological data were used in conjunction with a dispersion model to generate mathematical predictions of aerosol strength at various sampler locations. The mean calculated survival of aerosolized bacteria (standard plate count) in the range 46 to 76 m downwind was 5.2%, and that of coliphage f2 was 4.3%.

THE FOLLOWING IS PRESENTED TO DEMONSTRATE THAT OUTFALL PLACEMENT MAY HAVE A PROFOUND IMPACT ON BEACH CONTAMINATION.

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Several researchers have pointed out that wastewater, treated or untreated, is a primary contributor of bacteria to the aquatic ecosystem (12, 16, 17, 20, 27, 29). Studies have been conducted which demonstrate that significant numbers of multiple drug-resistant coliforms occur in rivers (17), bays (9), bathing beaches (28), and coastal canals (13). Waters contaminated by bacteria capable of transferring drug resistance are of great concern since there is the potential for transfer of antibiotic resistance to a pathogenic species.

McGowan's abstract.....

Short and shallow marine sewer outfalls, a connection with the spread of antibiotic resistance?

Of the 49 sewer outfalls along the California coast, 17 could be considered as short and shallow which may increase marine and beach sand contamination with antibiotic resistant pathogens. Public beaches adjacent to these short shallow outfalls (SSO) may become reservoirs of antibiotic resistance. Studies of beach sand in lake and marine systems have demonstrated contamination with a variety of pathogens. Some studies specifically considered antibiotic resistant Staphylococcus aureus (MRSA) which now kills more Americans than AIDS, according to the CDC.

Beach-goers who dug in the sand or covered themselves with sand were, in the following week or two, more likely to have diarrheal illnesses from a variety of organisms.

Beach sand, especially if it contains ground kelp, offers a good medium for the regrowth and maintenance of pathogens. Do SSO's augment the contamination of coastal beaches and are the bacteria likely to contain antibiotic resistant genetic material? An answer may be gained by tracking effluent released from SSO's. Pilot data indicate effluent may be returned to the beach and near shore waters.

Sewer plants are a principal source for the generation and release of antibiotic resistant bacteria and their genetic material, per peer reviewed published facts for approximately 40 years. US/EPA also published on the topic in 1982. The California Department of Public Health and the Regional Water Quality Control Boards do not recognize this potentially dangerous situation and consequently have generated no standards for its control.

CDC, US/EPA and IATFAR currently plan no research on the subject.

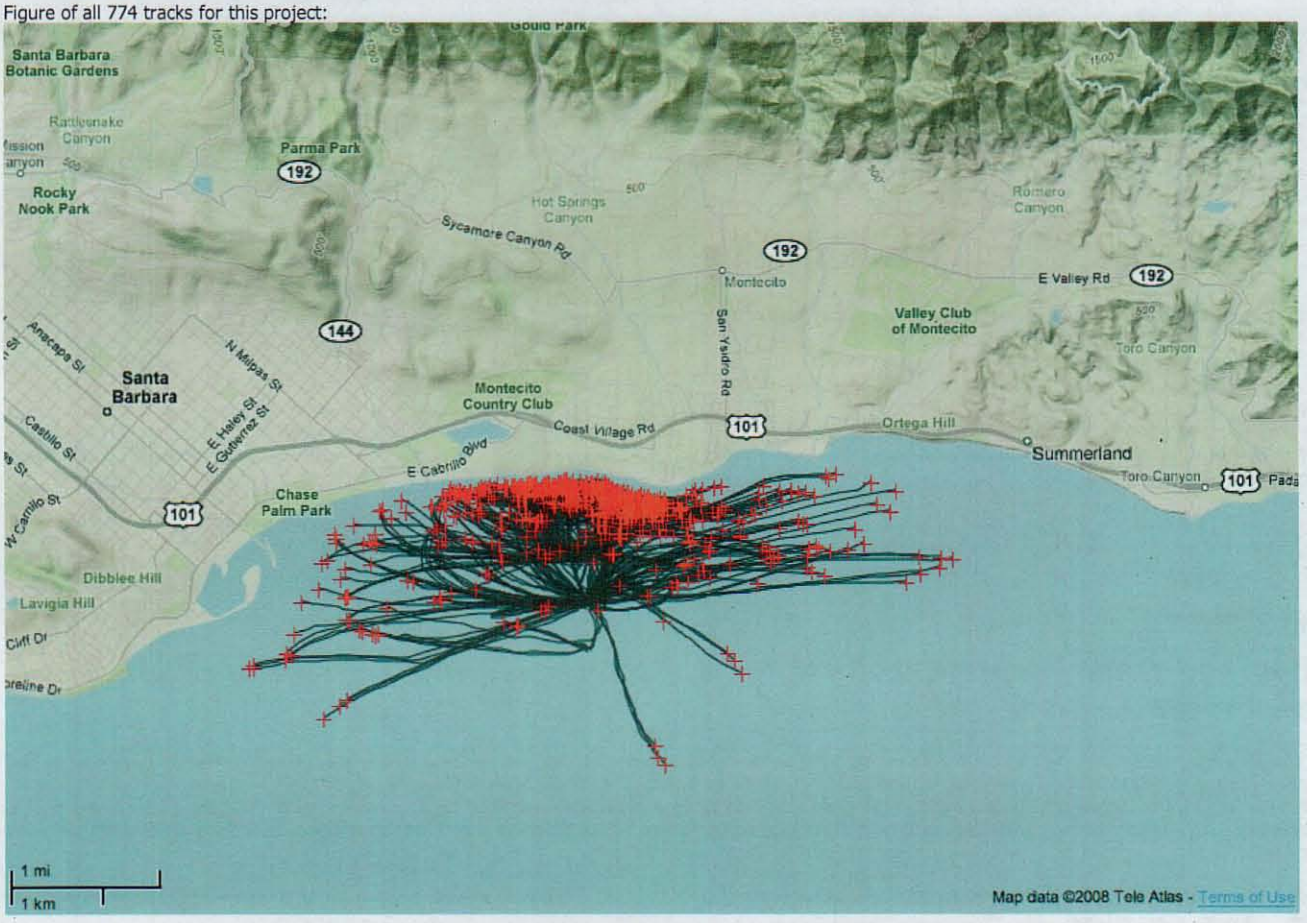
Table of California sewer outfalls which discharge less than ½ mile from shore and depth of less than 40 feet

POTW/WWTP .....Miles from shore..... Depth in feet

Anchor Bay 0.00 0
Shelter Cove 0.00 0
Fort Bragg 0.12 20
Crescent City 0.13 10
Eureka 0.00 0
Arcata 0.00 0
Half Moon Bay 0.36 37

Daly City	0.47	32
Ragged Pt Inn	0.00	0
Summerland	0.14	20
San Simeon	0.17	20
Avela Beach	0.42	29
Montecito	0.29	35
Carmel Area	0.11	35
Carpinteria	0.19	25
San Clemente Ils	0.00	0
Terminal Ils	0.17	32
Mendecino	0.19	60
Avalon	0.08	130

Figure of all 774 tracks for this project:



Trackers released at the subsurface discharge point of Montecito Sanitary District's sewer outfall showing movement back to beach area and stacking at beach-front. Using these data allows one to argue that released pathogens in the sewage effluent would follow tracker routes. Outfall is 0.29 miles from shore and at a depth of 35 feet. Black lines represent vector of tracker and red + is where tracker stopped its movement.

Exhibit 2

WHITE PAPER

AQUATIC LIFE CRITERIA FOR CONTAMINANTS OF EMERGING CONCERN

PART I

GENERAL CHALLENGES AND RECOMMENDATIONS

Prepared by the
OW/ORD Emerging Contaminants Workgroup

June 03, 2008

NOTICE

THIS DOCUMENT IS AN INTERNAL PLANNING DOCUMENT

It has been prepared for the purpose of Research & Development Planning. It has not been formally released by the U.S. Environmental Protection Agency and should not at this stage be construed to represent Agency guidance or policy.

DRAFT DOCUMENT

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List of Acronyms:

ACR	Acute to Chronic Ratio
ALC	Aquatic Life Criteria
Ah	Aryl Hydrocarbon (receptor)
AV	Acute (toxicity) value
AWQC	Ambient Water Quality Criteria
CCC	Criterion Continuous Concentration
CEC	Contaminants of Emerging Concern
CMC	Criterion Maximum Concentration
CV	Chronic (toxicity) value
CWA	Clean Water Act
CYP	Cytochrome enzymes (P450)
EDC	Endocrine Disrupting Chemicals
E2	Estradiol (natural estrogen)
EE2	Ethynylestradiol (synthetic pharmaceutical estrogen)
ELS	Early Life-Stage (toxicity test)
EPA	Environmental Protection Agency
FAV	Final Acute Value
FACR	Final Acute to Chronic Ratio
GMAV	Genus Mean Acute Value
GMCV	Genus Mean Chronic Value
HPG	Hypothalamic-Pituitary-Gonadal (axis)
HPT	Hypothalamic-Pituitary-Thyroid (axis)
LOEC	Lowest Observed Effect Concentration
MDR	Minimum Data Requirement
MOA	Mode of Action
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Development and Cooperation
OWC	Organic Wastewater Contaminant
PBDE	Polybrominated Diphenyl Ether
PPCP	Pharmaceutical and Personal Care Product
POP	Persistent Organic Pollutant
SMAV	Species Mean Acute Value
TBT	Tributyltin
USGS	United States Geological Survey
VTG	Vitellogenin protein
vtg	Vitellogenin gene transcript
WWTP	Wastewater Treatment Plant

1.0 INTRODUCTION

Under the United States Clean Water Act (CWA) (33 U.S.C. Sections 1251-1387), EPA is required to take a number of actions to protect and restore the ecological integrity of the Nation's water bodies. Under Section 304(a) of the CWA, EPA must develop and publish ambient water quality criteria. Ambient water quality criteria (AWQC) are levels of individual pollutants, water quality characteristics, or descriptions of conditions of a water body that, if met, should protect the designated use(s) of the water. Examples of designated uses of a water body include swimming, drinking water, fishing, fish spawning, and navigation. States and authorized tribes establish designated uses for their water bodies. AWQC are recommended guidance that states and tribes may use as part of their water quality standards to protect water bodies for their designated use from chemical pollutants.

AWQC for aquatic life (aquatic life criteria, ALC) developed under Section 304(a) reflect the "latest scientific knowledge" concerning "all identifiable effects" of the pollutant in question. These criteria are based solely on data and scientific determinations on the relationship between environmental concentrations of the pollutant and its effects. Criteria do not consider social and economic impacts, or the technological feasibility of meeting the chemical concentration values in ambient water. Since the early 1980's, EPA has been developing ALC to protect aquatic organisms from chemical specific pollutants under Section 304(a) of the CWA. In 1985, EPA published *Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses* (hereafter referred to as the "*Guidelines*"; Stephan et al. 1985). The *Guidelines* has provided uniformity and transparency in the derivation methodology of ALC for a large number of compounds among several classes of chemicals. The majority of EPA's currently recommended ALC have been derived using the methods outlined in the *Guidelines*.

While the *Guidelines* remain the primary instrument the Agency uses to meet its broad objectives for the development of ALC, there have been many advances in aquatic sciences, aquatic and wildlife toxicology, population modeling, and ecological risk assessment that are relevant to deriving ALC. Some of the advances have been addressed through supplemental guidance on the derivation or site-specific modification of criteria (Prothro 1993; U.S. EPA 1994a), while others have been incorporated directly into derivation of individual ALC for certain chemicals (e.g., saltwater chronic aquatic life criterion for tributyltin, U.S. EPA 2003). Recently, considerable attention has been generated by a widely ranging group of chemicals termed, in this document, contaminants of emerging concern (CECs). As is discussed in the body of this document, some of these CECs present challenges for the application of the *Guidelines* to ALC development.

1.1 What is a Contaminant of Emerging Concern?

The term "contaminant of emerging concern" is being used within the Office of Water to replace "emerging contaminant," a term that has been used loosely since the mid-1990s by EPA and others to identify chemicals and other substances that have no regulatory standard, have been recently "discovered" in natural streams (often because of improved analytical chemistry detection levels), and potentially cause deleterious effects in aquatic life at environmentally

relevant concentrations. They are pollutants not currently included in routine monitoring programs and may be candidates for future regulation depending on their (eco)toxicity, potential health effects, public perception, and frequency of occurrence in environmental media. CECs are not necessarily new chemicals. They include pollutants that have often been present in the environment, but whose presence and significance are only now being evaluated.

CECs include several types of chemicals:

- Persistent organic pollutants (POPs) such as polybrominated diphenyl ethers (PBDEs; used in flame retardants, furniture foam, plastics, etc.) and other global organic contaminants such as perfluorinated organic acids;
- Pharmaceuticals and personal care products (PPCPs), including a wide suite of human prescribed drugs (e.g., antidepressants, blood pressure), over-the-counter medications (e.g., ibuprofen), bactericides (e.g., triclosan), sunscreens, synthetic musks;
- Veterinary medicines such as antimicrobials, antibiotics, anti-fungals, growth promoters and hormones;
- Endocrine-disrupting chemicals (EDCs), including synthetic estrogens (e.g.,17α-ethynylestradiol, which also is a PCPP) and androgens (e.g., trenbolone, a veterinary drug), naturally occurring estrogens (e.g.,17ß-estradiol, testosterone), as well as many others (e.g., organochlorine pesticides, alkylphenols) capable of modulating normal hormonal functions and steroidal synthesis in aquatic organisms;
- Nanomaterials such as carbon nanotubes or nano-scale particulate titanium dioxide, of which little is known about either their environmental fate or effects.

1.2 Why is EPA Concerned with CECs?

The variety of chemicals labeled as CECs leads to a variety of concerns for EPA. Widespread uses, some indication of chemical persistence, effects found in natural systems, and public concerns have made clear the need for EPA to develop criteria that can be used to help assess and manage potential risk of some CECs in the aquatic environment. A recent U.S. Geological Survey (USGS) reconnaissance study (Kolpin et al. 2002) provides a good example of the prevalence of a wide range of CECs in U.S. streams. Improved analytical chemistry techniques were used to document the occurrence of what the authors called organic wastewater contaminants (OWCs) being released into surface waters from wastewater treatment plants (WWTPs). The targeted OWCs included PPCPs, veterinary medicines and other EDCs. The investigators found at least one of 95 different target OWCs in 80 percent of the 139 streams sampled. A median of seven, and as many as 38, OWCs were found in single samples.

The use and occurrence patterns associated with CECs are varied. Some CECs are similar to conventional toxic pollutants in that they are associated with industrial releases, whereas many others are used by the general public every day in homes, on farms, by businesses and industry (Daughton and Ternes 1999). PPCPs acting as EDCs can be released directly to the environment after passing through wastewater treatment processes, which are typically not designed to remove these pollutants from the effluent (Halling-Sorensen et al. 1998). Sludge from secondary treatment processes are land-applied as biosolids, supplying CECs which may leach or run off into nearby bodies of water. Pharmaceuticals used in animal feeding operations may be released

to the environment in animal wastes via direct discharge of aquaculture products (i.e., antibiotics), the excretion of substances in animal urine and feces of livestock animals, and the washoff of topical treatments from livestock animals (Boxall et al. 2003).

EDCs discharged at WWTPs are one group of CECs with potentially widespread environmental effects (Folmar et al. 1996; Folmar et al. 2001; Jobling et al. 1998; Woodling et al. 2006). Although particular concern has been expressed about the anthropogenic EDCs, there are also natural estrogens (estradiol and its metabolites estriol and estrone) entering the aquatic environment through wastewater discharge and excretion from domestic animals. Furthermore, little is known about the environmental occurrence, fate and, transport for any of these compounds after they enter aquatic ecosystems. Many of the man-made compounds have been in use for a long time, and there is concern about pharmacologically active ingredients and personal care products that are designed to stimulate a physiological response in humans, plants, and animals (Daughton and Ternes 1999).

Frequent detection of compounds by itself does not constitute a need for ALC. Rather, criteria development for CECs needs to focus efforts on chemicals that demonstrate a reasonable potential to adversely affect aquatic life. Of CECs now known to be found in some surface waters of the U.S., EDCs have received the most attention because field studies from around the world have demonstrated that very low concentrations of some of these compounds can significantly impact natural populations of aquatic vertebrates. For example, observational field studies (Jobling et al. 1998) have shown a high occurrence of intersex (the presence of both male and female characteristics) in wild populations of a fish known as roach (*Rutilus rutilus*) in rivers in the United Kingdom that are downstream from WWTPs. Similar results have recently been reported for white sucker (*Catastomus commersoni*) in northern Colorado, U.S.A (Woodling et al. 2006). In a multiyear study by Kidd et al. (2007), the authors showed that environmentally relevant concentrations of ethynylestradiol, EE2, caused reproductive failure and near collapse of a natural fathead minnow population in an experimental lake, and also had deleterious effects on the reproductive biology of the pearl dace. These direct effects resulting in loss of forage fish have led to cascading effects on the lake trout population due to lack of prey (Kidd, personal communication). Researchers from the U.S. Geological Survey (USGS) have observed intersex and testis-ova (the presence of eggs in the testis) in bass species collected from the Potomac River and its tributaries in West Virginia, Maryland, and Washington DC, and also quantified EDCs in their blood (Blazer et al. 2007; Chambers and Leiker 2006). The occurrence of intersex fish in the Potomac River, as well as documented occurrence of this and related effects in other waters of the US and internationally, prompted Congressional hearings that were held in October 2006 to inquire about the "State of the Science on EDCs in the Environment," as well as EPA activities associated with EDCs.

1.3 Purpose and Organization of This White Paper

The purpose of this white paper is to provide general guidance on how criteria development for CECs could be facilitated through a supplemental interpretation of the *Guidelines*, with particular attention to PPCPs with an EDC mode of action (MOA). Section 2 of this part (Part I) of the white paper describes the *Guidelines* procedures and identifies several areas in which these

procedures could be modified to address potential limitations for deriving criteria for CECs. Section 3 expands upon the areas of concern with respect to specific toxicological characteristics of some CECs. Section 4 summarizes these concerns and provides recommendations that could aid in the development of criteria for CECs in a resource efficient manner that takes best advantage of existing knowledge. Part II of this white paper further describes these concerns and recommendations using data for the synthetic pharmaceutical estrogen EE2.

2.0 CURRENT ALC METHODOLOGY

The *Guidelines* specify various data and procedural recommendations for criteria derivation, and also define general risk management goals for criteria, which are to provide a high level of protection for aquatic communities and for important species in these communities. ALC are defined to consist of two concentrations – the Criterion Maximum Concentration (CMC), intended to protect against severe acute effects, and the Criterion Continuous Concentration (CCC), intended to protect against longer term effects on survival, growth, and reproduction. The CMC is used in criteria to limit peak exposures by requiring that 1-hour averages of exposure concentrations not exceed the CMC more often than once in three years on average. The CCC is used in criteria to limit more prolonged exposures by requiring that 4-day averages of exposure concentrations not exceed the CCC more often than once in three years on average.

The CMC and CCC are usually derived from laboratory toxicity test results using specific standard procedures described in the *Guidelines*, but the *Guidelines* also have general provisions for deviating from these procedures as warranted by available information. The following text will first give an overview of the data requirements and calculations in the standard procedures, and then discuss how these procedures might vary under the umbrella of the *Guidelines*.

2.1 Standard ALC Derivation Procedures

The CMC is determined based on available Acute Values (AVs) – median lethal concentrations (LC_{50}s) or median effect (for a severe acute effect such as immobilization) concentrations (EC_{50}s) from aquatic animal acute toxicity tests (48- to 96-hours long) meeting certain data quality requirements. To compute a CMC, the *Guidelines* require that acceptable AVs be available for at least eight genera with a specified taxonomic diversity, in order to address a wide variety of the organisms constituting an aquatic animal community. These minimum data requirements include three vertebrates (a salmonid fish, a fish from a family other than salmonidae, and a species from a third chordate family) and five invertebrates (a planktonic crustacean, a benthic crustacean, an insect, a species from a phylum other than Chordata or Arthropoda, and a species from another order of insect or a fourth phylum).

For each genus, a Genus Mean Acute Value (GMAV) is calculated by first taking the geometric mean of the available AVs within each species (Species Mean Acute Value, SMAV) and then the geometric mean of the SMAVs within the genus. The fifth percentile of the set of GMAVs so obtained is calculated based on a specified estimation method, and designated the Final Acute Value (FAV). The FAV is then lowered to the SMAV for an important, sensitive species if appropriate. The CMC is set equal to half of the FAV to represent a low level of effect for the fifth percentile genus, rather than 50% effect.

The CCC is generally determined based on available Chronic Values (CVs), which are either (a) the geometric mean of the highest no-observed-effect concentration (NOEC) and lowest

observed effect concentration (LOEC) for effects on survival, growth, or reproduction in aquatic animal chronic tests or (b) in some recent criteria (e.g., ammonia), the EC_{20} in such tests based on concentration-effect regression analyses. Chronic tests for invertebrate species are required to include the entire life-cycle, but for fish partial life-cycle and early life-stage (ELS) testing protocols are accepted, the latter not including reproductive endpoints and not used if life-cycle or partial life-cycle tests are available and show more sensitive adverse effects.

If CVs are available for at least eight genera with the required taxonomic diversity, the CCC is set to the fifth percentile of Genus Mean Chronic Values (GMCVs), by the same procedure used to derive an FAV from GMAVs. Otherwise, the CCC is set to the FAV divided by a Final Acute Chronic Ratio" (FACR) that is based on acute to chronic ratios (ACRs – the ratio of the AV to the CV from parallel acute and chronic tests) for at least three species with a specified taxonomic diversity. The CCC can also be based on plant toxicity data if aquatic plants are more sensitive than aquatic animals, or on other data as deemed scientifically justified.

Further details on test requirements and calculation methods for the CMC and CCC are specified in the *Guidelines*, including deriving criteria that are a function of water quality characteristics.

2.2 Alternatives for ALC Derivation

The procedures described above enable broad application to toxic chemicals generally, and are only constrained by specific data requirements for quality and minimum taxonomic representation. Since they are not restricted with respect to specific types of chemicals, there is no reason to suppose that the standard data requirements and procedures specified by the *Guidelines* are any more or less applicable to CECs than to the chemicals for which criteria have already been developed. The *Guidelines* anticipated that rote application of the basic procedures may not yield the most appropriate criteria; consequently, the *Guidelines* provide flexibility when appropriate for deviation from the normal procedures regardless of the type of chemical, as indicated by the following provisions (hereafter referred to as the "Good Science" clauses:

> *"These National Guidelines should be modified whenever sound scientific evidence indicates that a national criterion produced using these Guidelines would probably be substantially overprotective or underprotective of aquatic organisms and their uses on a national basis."* (p. 18).
>
> *"On the basis of all available pertinent laboratory and field information, determine if the criterion is consistent with sound scientific evidence. If it is not, another criterion, either higher or lower, should be derived using appropriate modifications of these Guidelines."* (p. 57).

In addition, although the standard procedures in the *Guidelines* for deriving a CMC and CCC use only toxicity tests meeting certain requirements, the *Guidelines* also mandate the collation and examination of other data that might show effects that should be considered in criteria derivation:

> *"Pertinent information that could not be used in earlier sections might be available concerning adverse effects on aquatic organisms and their uses. The most important of these are data on ... any other adverse effect that has been shown to be biologically important. Especially important are data for species for which no other data are available. ... Such data might affect a criterion if the data were obtained with an important species, the test concentrations were measured, and the endpoint was biologically important."* (p. 54).

While alternatives are allowed when a specific situation dictates, the *Guidelines* still require that any changes in the procedures are consistent with the level of protection represented by the standard procedures:

> *"Derivation of numerical national water quality criteria for aquatic organisms and their uses is a complex process and requires knowledge in many areas of aquatic toxicology; any deviation from these Guidelines should be carefully considered to ensure that it is consistent with other parts of these Guidelines."* (p. iv).

Therefore, for the development of criteria for any chemical, the general strategy should be to start with the standard *Guidelines* procedures and then to adapt those procedures as warranted by available information on the effects of the chemical. This strategy applies to CECs as well, although certain considerations might more consistently be important for CECs. Specific attributes of CECs that might affect criteria derivations are considered in Section 3 of this paper, but several issues are introduced here that are of general concern.

Are data on acute toxicity needed for risk assessments?

Some chemicals are not acutely toxic even at concentrations so high that they could not possibly occur in the environment (e.g., at the chemical solubility, or exceeding exposures possible based on known chemical production and discharges). The acute lethality of some classes of chemicals might be measurable, but would occur at environmental concentrations so much higher than those affecting reproduction, growth, or chronic survival that, in practice, environmental exposures will always be far below acutely lethal levels if those exposures are managed to limit chronic effects. Therefore, derivation of the CMC might be unnecessary or impossible. Thus, if the existing data indicate that it is reasonably certain that acute toxicity would not occur at environmentally relevant concentrations, conducting additional acute tests is likely to be unwarranted.

Even if a CMC is not needed, another use of acute toxicity data is for developing "acute to chronic ratios" (ACRs) that are used with the FAV to calculate the CCC (see pages 40-42 in the *Guidelines*) , so that dropping acute testing requirements must consider this consequence as well. However, if acute effect concentrations are extremely high compared to chronic effect concentrations (large ACRs), whether the ACR approach should be even used warrants some consideration. Large ACRs are not, *per se*, less accurate than low ACRs, provided acute and chronic effect concentrations are well defined and the issue is simply extrapolating from acute to chronic toxicity within a species. However, for criteria calculations, the FACR needs to be a ratio that extrapolates from the fifth percentile of the acute effect concentration distribution to the fifth percentile of the chronic effect distribution. This requires appropriately combining ACR

information across species, the accuracy of which might be affected by large ACRs even if the accuracy of the individual ACRs is not. Therefore, in addition to not needing a CMC, extreme acute tolerance might also warrant direct calculation of the CCC rather than using the ACR approach, and thus eliminate the need for fulfilling all of the minimum acute toxicity test requirements as specified by the *Guidelines*.

How should data requirements for tolerant taxa be addressed?
The fifth percentile calculation methods for the CMC (as well as the CCC if the eight minimum data requirements noted above are met) require actual GMAV (or GMCV) values only for the four most sensitive genera. For more tolerant genera, it is only necessary to know that these toxicity values are greater than those of the four sensitive species. Therefore, toxicity test results that report "greater than" effect concentrations are acceptable for the tolerant taxa, and in fact are used in various criteria already.

If chronic tests have not already been done on some taxa needed for the minimum data requirements, but which are known to be tolerant, testing resources might be wasted by generating numbers that will not affect results. If methods such as inter-chemical or inter-species extrapolation methods, or assays (e.g., *in vitro* tests, biomarkers) that have been related to apical effects such as reductions in growth, survival, or reproduction can demonstrate these taxa to be insensitive compared to other taxa, actual chronic tests on these taxa may not be needed. In other words, can minimum data requirements for tolerant taxa be satisfied by some type of estimation rather than by an actual test result?

However, adding estimated data can become a rather open-ended process. Therefore, consideration must be given to how many estimated values should be allowed, relative to measured values, to produce an appropriate distribution of taxa in the data set used for criteria derivation.

Should fish chronic tests be required to address reproduction?
For chemicals (e.g., environmental estrogens) for which reproductive toxicity is of most concern, the allowance in the *Guidelines* for using ELS tests might need reconsideration. The *Guidelines* already give priority to life-cycle and partial life-cycle tests when they are available and show greater sensitivity than ELS tests. However, other information (from other species, similar chemicals, knowledge of the MOA) regarding latent or multigenerational reproductive effects may demonstrate the importance of sexual development and reproduction, so as to establish a basis for not considering ELS test results (or even partial life-cycle tests), but rather requiring life-cycle tests for fish.

What endpoints can serve as surrogates for traditional chronic endpoints?
Although chronic criteria are and will continue to be based on effects on reproduction, growth, and survival, another issue is whether only toxicity data directly addressing these endpoints is acceptable. Is there additional information (e.g., sub-organismal biomarkers, behavioral data) that can be used in criteria derivation because they are adequately correlated to reproduction, growth, and survival? Use of such data would be consistent with the *Guidelines* requirements to examine all pertinent data and make modifications to the criteria derivation procedures that are consistent with sound scientific evidence

2.3 Precedent for Deviating from Basic ALC Derivation Procedures

The recent ALC document for tributyltin (TBT) provides a good example of some of the types of procedural criteria modifications discussed above. TBT is a highly toxic biocide that has been used extensively in anti-fouling paint to protect the hulls of large ocean-going ships. It is deemed a problem in the aquatic environment because it is extremely toxic to non-target organisms, and has been linked to imposex (the superimposition of male anatomical characteristics on females) and to immuno-supression in snails and bivalves (U.S. EPA 2003). The concentrations reported to cause imposex in the laboratory are lower (range: 0.0093 to 0.0334 µg/L) than the FCV (0.0658 µg/L) calculated using the standard ALC derivation procedures (U.S. EPA 2003). The low effect concentrations established for female gastropods in the laboratory were subsequently corroborated in field studies. The CCC was lowered (to 0.0074 µg/L) based on the judgment that these effects were relevant for the risks of TBT to gastropod reproduction.

3.0 CHARACTERISTICS OF CECS AND THEIR INFLUENCE ON ALC DEVELOPMENT

As described in Section 1.0, chemicals become labeled as CECs for a variety of reasons, many of which have relatively little to do with their toxicological characteristics. Consequently, the Guidelines cannot be interpreted or modified in one particular way that would be universally appropriate for all CECs. However, some characteristics may be shared by various CECs, such that discussing the implications of these characteristics in the context of deriving water quality criteria is worthwhile. The expected outcome is additional guidance addressing key issues that may arise and how best to accommodate these issues in deriving criteria.

Much of the technical discussion that follows is centered on EDCs and, even more specifically, around chemicals that interact with the hypothalamic/pituitary/gonadal (HPG) axis. Endocrine function controlled via the HPG axis involves hormones broadly known as estrogens ("female" hormones such as estradiol) and androgens ("male" hormones such as testosterone), along with the body tissues and biochemical machinery with which they interact. Effects on this part of the endocrine system of various aquatic species have been documented in the literature, and publicized in the media, making toxicological disruption of this mechanism a good choice for discussing CECs in the context of the *Guidelines*. However, these types of substances are only a subset of EDCs, and an even smaller subset of CECs as a whole. While much of the discussion that follows uses HPG-active chemicals as a point of reference, the concepts presented may be useful in the derivation of ALC for many other chemicals as well. It is the principles more than the specifics that are important in considering the content of this report.

3.1 Characteristics of HPG-Active EDCs

While estrogenic and androgenic hormones are a core component of the HPG axis, this system also includes a much larger group of tissues and biochemical machinery within the body which, in vertebrates, govern sexual development, maturation, and reproduction. Commensurate with this complexity, there are many places within the system that environmental chemicals may act to modify the normal function of the HPG-axis. Thinking simply of "estrogenicity" or "androgenicity" as toxicological modes of action is still too broad – these categorical classes are more the outward "symptoms" of disruption in the HPG axis than they are unique modes of toxicological action. For example, the synthetic steroids EE2 and trenbolone bind to (and act as agonists of) vertebrate estrogen and androgen receptors, respectively, with similar or greater affinity than the natural endogenous hormones, estradiol and testosterone. In contrast, a variety of other medicinal pharmaceuticals are specifically designed to do the opposite, to be antagonists of these same receptors. As examples, tamoxifen (breast cancer treatment) and flutamide (prostate cancer treatment) bind quite specifically to vertebrate estrogen and androgen receptors, respectively, thereby blocking the activity of endogenous steroid hormones. But disruptors of the HPG axis are not limited to chemicals that bind directly to estrogen or androgen receptors; they also include chemicals that interact elsewhere in the overall biochemical pathway. As an example, there are chemicals that exert their activity through interactions with CYP (cytochrome

P450) enzymes involved in steroid production. The pharmaceutical chemical fadrozole acts to inhibit CYP19 aromatase, the enzyme that converts estradiol to testosterone. A number of conazole fungicides act as competitive inhibitors of several CYPs further up the steroid synthesis pathway (Ankley et al. 2005).

Unlike many other chemicals that have either non-specific (e.g., narcotics) or more generalized reactive modes of action (e.g., electrophilic chemicals interacting with nucleic acids and proteins), HPG-active compounds tend to have very specific interactions with particular molecular targets within the biochemical pathway. There are a number of consequences arising from this specificity. One important consequence of target specificity is potency. Many pharmaceuticals are designed to be highly specific, and thus are extremely potent. For example, EE2 and trenbolone affect reproduction and development in fish at water concentrations in the very low ng/L (part-per-trillion) range (e.g., Ankley et al. 2003; Länge et al. 2001), well below effect concentrations for most chemicals for which current ALC have been derived. These very low biologically-active concentrations present substantial challenges for analytical determinations associated either with lab-based effects testing or field monitoring of *in situ* exposures. In the case of EE2 and/or trenbolone, effects observed in fish are at concentration levels below the methodological limit of detection for most laboratories even in laboratory test water, and even more so ambient water and effluents.

Such high potency can influence how one would approach criteria derivation when the chemicals exert minimal acute toxicity, but cause mostly long-term, sub-lethal effects. Trenbolone and EE2 illustrate this situation quite well. Like most pharmaceuticals (some exceptions being chemotherapy and anti-parasitical agents), these chemicals are designed to "adjust" the biochemistry of the body without causing acute toxicity or other significantly adverse side effects. As a consequence, these types of pharmaceuticals tend to have low toxicity in short-term lethality assays (Webb 2001). In the context of criteria development, this has implications for the use of ACRs. Most conventional toxic pollutants with EPA ALC have ACRs of 10 or less (Cunningham et al. 2006; Host et al. 1995). In contrast, ACRs for EE2 and 17β-trenbolone in fish have been shown to range from 1,000 to greater than 300,000 (Ankley et al. 2005). Again, this is a result of relatively low acute toxicity and high chronic potency. Importantly, limited data for other MOA classes of pharmaceuticals suggest that this phenomenon is not restricted to endocrine-active substances. For example, Huggett et al. (2002) reported an ACR in fish of about 50,000 for propanolol, a β-blocker. As discussed in Section 2, this large difference in acute and chronic potency may both make CMC values moot in the environmental management of these chemicals, and introduce uncertainty in the extrapolation between acute and chronic effects in the derivation of a CCC.

The specificity of the molecular target also can greatly affect those taxa likely to be sensitive to the chemical MOA of concern. While some biological pathways (e.g., energy metabolism) tend to be highly conserved across all organisms, others can be quite specific to certain phylogenetic groups. Although the control of reproductive function through the HPG axis is highly conserved across vertebrate classes, lower taxonomic groups such as invertebrates have different endocrine system structure that function differently. For example, Segner et al. (2003) tested several estrogenic chemicals, including EE2, in a variety of partial and full life-cycle tests with a model fish (zebrafish) and several aquatic invertebrate species. They found that the fish was by far most

sensitive to the effects of the estrogenic chemicals, and was the only species that responded to EE2 at environmentally-relevant concentrations. As a result, it is likely that chronic toxicity data for fish would be the most influential in setting the criterion for EE2, and correspondingly unlikely that toxicological data for invertebrate species would have much impact. Plants do not have comparable endocrine system structure or function, and would not be expected to be sensitive to these types of compounds, but there is research that indicates that algal species may be a uniquely sensitive taxa for the assessment of other types of CECs such as antibacterial products like triclosan (Orvos et al. 2002; Wilson et al. 2003).

Specificity in MOA can also affect how or if effects are expressed within a toxicity test, even in potentially sensitive species. In the case of chemicals that affect endocrine function, there are distinct "windows" when animals are likely to be sensitive and/or exhibit adverse outcomes. For example, a popular amphibian early developmental assay-FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) would be inadequate for detecting thyroid-active toxicants because the period of exposure and observation occurs early in development before the thyroid axis is functional in *Xenopus*. In the case of HPG-active toxicants, there are two windows of sensitivity during an animal's life: during sexual differentiation in developing organisms (when "organizational" alterations occur), and during active reproduction in adults (when "activational" responses can be manifested; Ankley and Johnson 2004). As a result, it is critical that testing for HPG-active EDCs occur during periods when the system is vulnerable to disruption. It is equally critical that toxicity tests include observations during the periods when effects are expressed. Some of the changes caused during sensitive early developmental windows may not be expressed until later in life. For example, the ELS toxicity test protocol commonly used in criteria development to estimate the chronic sensitivity of fish contains the early life stages that could be sensitive to disruption of sexual development, but it does not extend through to maturation, and would therefore be insensitive for detecting disruption of sexual development.

3.2 Implications for ALC Development

As is clear from the text above, some characteristics of HPG-active chemicals (and many other CECs) create the need to carefully interpret the intent of the *Guidelines*, not just the routine derivation process. As indicated in Section 2.0 there is nothing about CECs that invalidates the principles embodied in the *Guidelines*; however, the *Guidelines* were written before many of the issues discussed in Section 3.1 were known, so they do not necessarily contain prescriptive guidance for some of the nuances created by some CECs. The following paragraphs discuss the implications of these issues for criteria development, following the four general topic areas outlined in Section 2.0:

- The need for and relevance of acute toxicity data and a CMC;
- Defining minimum data requirements in terms of taxonomic coverage;
- Defining appropriate chronic toxicity data; and
- Selecting effect endpoints upon which to base criteria

3.2.1 The Need For and Relevance of Acute Toxicity Data and a CMC

As described in Section 2.2, there are two primary uses for acute toxicity data under the *Guidelines*: 1) the derivation of the CMC; and 2) establishment of the CCC when the FAV/FACR method is used. As a practical matter, if the CMC is more than 96-fold higher than the CCC, then it will always be the CCC that is more limiting. This is because in the standard formulation of criteria, the CMC has a one-hour averaging period and the CCC a 96-hour averaging period; thus, if the difference between them is more than 96-fold, it is mathematically impossible to exceed the CMC without also exceeding the CCC. A minor exception to this issue occurs when ALC are implemented in an NPDES permit such that the CMC is applied to whole effluent while the CCC is applied after mixing, and the available in-stream dilution is large. However, these exceptions are rare, and even the 96-fold difference discussed here pales in comparison to the factors of 1,000 to 300,000-fold discussed previously in regard to EE2 and trenbolone. In cases where such extreme differences between acute and chronic toxicity thresholds exist, establishing ALC as having only a CCC seems a reasonable approach.

While it is easy to see why a CMC would not be necessary when you have sufficient acute test data to show that the CMC would be dramatically higher than the CCC, this begs the question of how much data are needed to decide that this is the case. This decision should occur during the "problem formulation" step in the risk assessment for a specific chemical/class, and should be guided by the following types of information:

- the amount and phylogenetic spread of acute toxicity data that are available;
- toxicity data from short-term exposures that do not meet the strict definitions in the *Guidelines* of acute toxicity data acceptable for criteria derivation, but from which information on responses to acute exposures can be inferred;
- short-term effect data garnered from longer-term exposures;
- information from closely related chemicals that are thought to have the same MOA, and have more robust acute data sets; and
- knowledge of the degree of phylogenetic distribution of the toxicity pathway of concern.

A complicating issue resulting from a "moot" CMC is that data availability for acute effects will likely be limited. As such, having less than the required acute MDRs may preclude the ability to derive a CCC using the FAV/FACR approach typically used in the *Guidelines*. For chemicals with highly specific modes of action and large ACRs (such as many EDCs), it is very likely that the mechanisms for acute and chronic toxicity differ, since biological activity resulting in chronic effects is designed into the product and not a secondary consequence - such as many of the historical contaminants for which EPA has developed criteria. Also, sensitivity of different taxa classes to acute and/or chronic toxicity varies widely due to presence (or absence) and structure and function of phylogenetically-conserved systems. Both of these issues would introduce considerable uncertainty into the availability and interpretation of ACRs, and probably make it inadvisable to use the FAV/FACR approach anyway. The *Guidelines* discuss the inadvisability of using the ACR approach when ACRs vary by more than a factor of 10 without a clear relationship to taxonomy or acute sensitivity (page 41 of the *Guidelines*). A more advisable approach would generally be to develop a CCC directly from a sufficiently robust set of chronic data, using the procedures outlined in the *Guidelines* or an appropriate modification thereof.

3.2.2 Defining Minimum Data Requirements in Terms of Taxonomic Coverage

To develop a CCC directly from chronic toxicity data (rather than via FAV/FACR), the *Guidelines* require that acceptable chronic toxicity data be available from at least eight families with a taxonomic distribution fulfilling the requirements specified in the *Guidelines* (referred to as the "minimum data requirements" or "MDRs"). Having a blanket requirement for meeting the eight MDRs was included to insure a minimum level of "certainty" that the *Guidelines* will be protective of the broad phylogenetic distribution of organisms found in aquatic systems. Including this phylogenetic spread also enables criteria to be developed for chemicals for which the toxicological MOA is not known. Instead of "knowing" what organisms are most likely to be sensitive to a particular chemical, requiring a broad spread of empirical toxicity data makes it likely that whatever taxa may be sensitive to a chemical, they will be represented to some degree in the toxicity data set.

In the case of EDCs, PPCPs, and certain other chemical classes, we may have a reasonable understanding of the toxicological MOA for the chemical, and from that knowledge we may be able to infer what taxa are most likely to be sensitive to a particular chemical (Ankley et al. 2007; Williams 2005). As discussed in Section 2.2, the procedure used in the *Guidelines* for estimating the 5^{th} percentile of a toxicity distribution is dependent on only the four lowest values; for this reason exact values for insensitive genera are not necessary, as long as there are sufficient data to infer that their sensitivity is lower than the four most sensitive genera.

So how does one determine that a particular taxon is insensitive? The general structure of the *Guidelines* presumes that sensitivity is determined by conducting an acceptable toxicity test with that taxon. However, one can infer that the actual need is only to have sufficient information to conclude that the taxon is insensitive; if that determination can be confidently made based on other information, the information need may be met even if an actual toxicity test with that particular organism and chemical has not been conducted. This does not change the intent of the *Guidelines*. It only acknowledges the possibility that there is more than one way to meet the information requirement.

Using the example of EE2, there is both physiological understanding and some empirical toxicity data to support the belief that vertebrates will be far more sensitive to EE2 toxicity than will invertebrates (see Part II of this white paper and Segner et al. 2003). As such, it would seem inappropriate to invest resources in testing a wide range of invertebrate taxa classes for sensitivity when all existing data indicate that the data would not affect the final criterion, which would be driven by sensitivity of vertebrates. In this case, it makes sense to argue that certain of the eight MDRs might be declared met not through direct testing, but through toxicological understanding of the chemical's MOA and the physiology of those other taxa or existing toxicity data that establishes sensitivity differences among taxa.

While this logic is clear, one must be careful in presuming that the primary MOA demonstrated by organisms with the target physiology is the only toxic MOA for the chemical. Particularly given the phylogenetic diversity of organisms, it is always possible that a chemical that behaves

with one MOA in one class of organisms may exert toxicity through a different mechanism in a different phylogenetic group. There are precedents for this scenario (Ankley et al. 2007). For example, exposure to the non-steroidal anti-inflammatory drug diclofenac via consumption of dead livestock has greatly diminished some populations of vultures in several East Asian countries. Diclofenac kills the birds through renal failure, which is only a relatively minor side-effect of the drug in mammals. While the mechanism of renal toxicity in vultures is likely molecularly related to the mechanism of therapeutic action in man, i.e., both seem to occur due to inhibition of prostaglandin synthesis (Meteyer et al. 2005), the inhibition of similar molecular components appears to be manifested as dramatically different whole organism endpoints. The key is in achieving a reasonable balance between expending resources on collecting data most likely to influence the criterion, while maintaining some kind of backstop against initially unexpected toxicity in other organisms.

One possibility for enhancing confidence regarding phylogenetic sensitivity is in considering data for other, closely-related chemicals with the same MOA. While the *Guidelines* focus analysis on toxicity data for the specific chemical in question, an understanding of toxicological MOA can also lead to an understanding of how other chemicals might act to exploit the same biological system in the same way. For example, one might reasonably infer that the relative species sensitivity to EE2 is likely similar to 17-ß estradiol (E2), the natural hormone which EE2 mimics. If, for example, there were a taxon which had been tested and found insensitive to E2, but had not been tested with EE2, it seems a reasonable assumption that that taxon would also be insensitive to EE2.

The possibility of fulfilling certain information requirements using data other than from direct toxicity testing does raise some other interpretation challenges, in particular the definition of the sample size for determining the 5^{th} percentile of the genera sensitivity distribution. For example, if one has reason to believe that all crustaceans would be insensitive to a chemical, how many genera does that assertion represent in the calculation of the genera sensitivity distribution? While this is a real issue that will have to be addressed, we believe the problem is tractable and the details of the resolution are left to later work.

Because of the risk that our mechanistic understanding of a chemical may be incomplete, it seems unlikely that one could justify completely bypassing several MDRs solely on theoretical arguments (e.g., developing a criterion for a testosterone mimic based only on chronic toxicity data for vertebrates, with no invertebrate data at all). At the same time, prudent application of other data types to fulfill certain information requirements for criteria derivation does seem appropriate. Given the tremendous variation in understanding and availability of data likely to exist for different CECs, it is presumed that at least initial application of this approach will have to be justified on a chemical-by-chemical basis using appropriate scientific judgment. However, lines of evidence that might be applicable to this determination include:

- an in-depth understanding of the toxicological (or, in the case of drugs, therapeutic) MOA;
- information on the basic physiology of other taxa in relation to the MOA;
- toxicity data from chronic exposures or other relevant experiments that do not meet the strict definitions of acceptable chronic data given in the *Guidelines*, but from which

information on relative taxon sensitivity can be inferred; and

- information from closely related chemicals that are thought to have the same MOA and have more robust acute or chronic data sets.

A separate, but related issue arises in respect to data from species not resident to North America. The *Guidelines* specify the use of toxicity data only from species resident to North America. However, particularly in regard to the study of EDCs, some fish species not resident to the U.S. have been advanced as experimental models for the evaluation of the chronic effects of EDCs to fish. Two clear examples at the time of this report are the zebrafish (*Brachydanio rerio*) and the Japanese medaka (*Oryzias latipes*), for which equivalency of EDC test data (with the fathead minnow) has been proposed through international groups such as the Organization for Economic Cooperation and Development (OECD; Ankley and Johnson 2004). These species have a rich toxicological database, and we know of no reason to believe that their sensitivities would be expected to be substantively different from sensitivities of at least some fish resident to the U.S. In keeping with international harmonization, we suggest that toxicity data from species with recognized international equivalency be included in criteria derivation with the full weight given to data from resident species.

3.2.3 Defining Appropriate Chronic Toxicity Data

The *Guidelines* state that acceptable chronic tests for criteria derivation are full life-cycle exposures (F_0 egg to F_1 offspring) for both vertebrates and invertebrates, as well as partial life-cycle (adult to juvenile) and early life-stage (ELS; egg to juvenile) tests for fish. The acceptance of ELS tests in particular as acceptable chronic tests is predicated on the work of McKim et al. (1978) and other evidence that the toxicity thresholds obtained from ELS tests are generally within a factor of two of the thresholds from life-cycle chronic tests.

While this general approach has been applied with apparent success for many chemicals, the *Guidelines* intimate concerns with the approach, noting that for some chemicals, ELS tests might not be good predictors of chronic toxicity, which would violate the principle underlying the use of ELS tests as chronic data (page 39 in the *Guidelines*). As noted previously, toxicological data for chemicals like EE2 show that certain chemicals may have potent effects on life processes that lie outside the exposure period represented by ELS tests (e.g., pronounced effects on reproduction), or on life processes for which the expression of effects does not occur until after the ELS period (e.g., embryo or larval exposure resulting in effects on sexual development and maturation in adult fish; see Section 3.1). It is clear from these examples that there are chemicals for which ELS tests should not be used as surrogates for full life-cycle exposures. In fact, chemicals that affect sexual differentiation may not be adequately assessed even with partial life-cycle exposures, since these protocols do not generally include observation of sexual development/maturation in fish exposed during early development.

While the "Good Science" clause and other text in the *Guidelines* would not support reliance on ELS tests as chronic data for chemicals known to have specific effects on other life processes, such as sexual development or reproduction, the *Guidelines* would allow the development of a criterion using only ELS data for fish if there were not any specific data to indicate that this approach would be inappropriate. This is akin to an "innocent until proven guilty" approach.

However, we believe experiences with chemicals like EDCs make clear the need to move from the previous approach to one of "guilty until proven innocent." In other words, it is probably wiser to require that the chronic toxicity data for fish include exposure and observation over a full life-cycle unless there is an affirmative reason to believe that it is not necessary (note: this issue is equally relevant to invertebrates species, but the ELS tests discussed in the *Guidelines* are focused explicitly on fish; invertebrate tests would already be required to be life-cycle). In keeping with this shift in emphasis, we believe the requirements for chronic toxicity data in the *Guidelines* should be tightened by adding the further requirements that either:

1) Full life-cycle data be available for at least one fish species; or

2) There is a body of experimental information indicating that life processes outside the ELS or partial life-cycle exposure/observation windows would not be important to capturing the important toxicological effects of the chemical.

At first glance, #2 may seem like requiring the proof of a negative, in that one would have to actually conduct the life-cycle test required by #1 in order to show that #2 is true. However, we believe there may be circumstances in which there may be data that speak to the sensitivity of different life stages that come from studies that, while scientifically valid, for some reason do not meet all the requirements of a valid life-cycle test as defined in the *Guidelines*. For example, there may be data for a life-cycle test with a non-resident species that includes the relevant life processes but does not qualify as an acceptable chronic test for the derivation of criteria because it is non-resident. Alternatively, there may be data from experiments that violate other requirements of acceptable toxicity data under the *Guidelines*, but still provide insight into sensitive exposure periods or life processes. Even though CVs from such data may not be used directly to calculate a chronic criterion, it seems reasonable to use such data to evaluate the question of where in the life-cycle there are important windows of exposure and/or effect and how that constrains the adequacy of ELS tests to represent chronic toxicity. In other cases, there may be sufficient information from other types of research to demonstrate to a reasonable level of certainty that a chemical's toxicological mechanism(s) would not preclude the use of ELS tests as indicators of chronic toxicity.

Where life-cycle toxicity data are available, the results of those experiments should be carefully examined to determine the likelihood that important windows of exposure or effect lie outside ELS test protocols. Obviously, if there is meaningful potential for effects outside the ELS exposure period, ELS tests should not be used as surrogates for more involved chronic tests. It may also be that the knowledge of toxicological mechanism for a particular chemical may be sufficient that meaningful chronic toxicity data could be developed from exposures that have a structure different from the life-cycle, partial life-cycle, and ELS protocols defined explicitly in the *Guidelines*. While defining such alternate protocols is beyond the scope of this document, we recognize the potential for such a situation and leave it to appropriate implementation of the "Good Science" clause to allow for inclusion of such alternative exposure protocols as surrogates for chronic toxicity data, most likely in addition to, rather than instead of, data from life-cycle toxicity tests.

At the other end of the spectrum lie toxicity tests that extend beyond the definition of a full life-

cycle test, often referred to as multi-generational tests. Because they encompass the full range of life processes as a life-cycle test, we feel that they should be included as acceptable chronic tests, assuming they meet all other requirements for test acceptability. Some studies have reported effects from EDC or other chemicals in which exposure to one generation creates effects in a later generation that were not observed in prior generations even at the same life stage (Nash et al. 2004). If substantial, such effects could create a situation where even full life-cycle toxicity tests might underestimate the chronic toxicity of a chemical and therefore produce criteria that are potentially under-protective. While we recognize the potential for this situation, at present we believe there is not sufficient reason to make multi-generational testing a requirement for criteria development, unless there is specific, compelling information that a criterion would be substantially under-protective if multi-generational effects were not rigorously considered.

3.2.4 Selecting Effect Endpoints Upon Which to Base ALC

The selection of endpoints appropriate to the derivation of ALC must be tied to the narrative intent of the overall *Guidelines*. The stated goal of ALC is to "protect aquatic organisms and their uses" (see *Water Quality Standards Handbook*; U.S. EPA 1994b). While the exact meaning of "protection" is not defined, there is considerable discussion in the *Guidelines* document that makes clear that protection does not mean the prevention of any measurable biological effect in any organism. Instead, there is discussion of endpoints that are "biologically important" and prevention of "unacceptable effects"; this implies that in the context of criteria there are effects that are "biologically unimportant" and/or levels of effect that are "acceptable." Related concepts include the idea that natural populations can withstand some magnitude/frequency of disturbance and still meet the intent of the *Guidelines*.

With "protection of aquatic organisms and their uses" as the assessment endpoint, a decision must be reached as to which biological responses (measurement endpoints in risk assessment parlance) are appropriate to address this goal. Survival, growth, and reproduction are processes that are generally accepted as being directly related to this goal, as these are all demographic parameters that directly affect population dynamics (although, the exact quantitative relationship is not always fully determined). However, there are many more biological responses that have been observed in response to toxicant exposure, both at the whole organism level (e.g., behavior) and at lower levels of biological organization (e.g., biochemical or histological changes). For many of these endpoints, their relationship to the assessment goal, "protection of aquatic organisms and their uses," is less clear. In this regard, we must consider an additional goal of the *Guidelines* – that criteria "provide a reasonable and adequate amount of protection, with only a small possibility of considerable overprotection or under-protection" (page 5 of the *Guidelines*). In keeping with this intent, it is important that criteria focus on endpoints that affect the assessment endpoint, but not create overprotection by preventing any measurable effect (or possibility of that effect). There must be a reasonable, affirmative connection between the measured response and the assessment endpoint.

The Agency's *Framework for Ecological Risk Assessment* (U.S. EPA 1992) identifies this problem:

> *In many cases, measurement endpoints at lower levels of biological organization may be more sensitive than those at higher levels. However, because of compensatory mechanisms and other factors, a change in a measurement endpoint at a lower organizational level (e.g., a biochemical alteration) may not necessarily be reflected in changes at a higher level (e.g., population effects).* (p. 14)

And later on:

> *Ideally, the stressor-response evaluation quantifies the relationship between the stressor and the assessment endpoint. When the assessment endpoint can be measured, this analysis is straightforward. When it cannot be measured, the relationship between the stressor and measurement endpoint is established first then additional extrapolations, analyses, and assumptions are used to predict or infer changes in the assessment endpoint.* (p. 23)

> *Measurement endpoints are related to assessment endpoints using the logical structure presented in the conceptual model. In some cases, quantitative methods and models are available, but often the relationship can be described only qualitatively. Because of the lack of standard methods for many of these analyses, professional judgment is an essential component of the evaluation. It is important to clearly explain the rationale for any analyses and assumptions.* (p. 23)

Existing criteria documents contain many types of data that were not used in the criteria derivation (the documents collate and review these data, but they are not used to actually define the criterion concentration) and it is useful to the discussion here to consider how such data have been interpreted. For example, the following text is derived from the most recent criteria document for ammonia (U.S. EPA 1999, see Appendix 5):

> *Endpoint indices of abnormalities such as reduced growth, impaired reproduction, reduced survival, and gross anatomical deformities are clinical expressions of altered structure and function that originate at the cellular level. Any lesion observed in the test organism is cause for concern and such lesions often provide useful insight into the potential adverse clinical and subclinical effects of such toxicants as ammonia. For purposes of protecting human health or welfare these subclinical manifestations often serve useful in establishing 'safe' exposure conditions for certain sensitive individuals within a population.*

> *With fish and other aquatic organisms the significance of the adverse effect can be used in the derivation of criteria only after demonstration of adverse effects at the population level, such as reduced survival, growth, or reproduction. Many of the data indicate that the concentrations of ammonia that have adverse effects on cells and tissues do not correspondingly cause adverse effects on survival, growth, or reproduction. No data are available that quantitatively and systematically link the effects that ammonia is reported to have on fish tissues with effects at the population level. This is not to say that the investigators who*

reported both tissue effects and population effects within the same research did not correlate the observed tissue lesions and cellular changes with effects on survival, growth, or reproduction, and ammonia concentrations. Many did, but they did not attempt to relate their observations to ammonia concentrations that would be safe for populations of fish under field conditions nor did they attempt to quantify (e.g., increase in respiratory diffusion distance associated with gill hyperplasia) the tissue damage and cellular changes (Lloyd 1980; Malins 1982). Additionally, for the purpose of deriving ambient water quality criteria, ammonia-induced lesions and cellular changes must be quantified and positively correlated with increasing exposures to ammonia.

In summary, the following have been reported:

1. Fish recover from some histopathological effects when placed in water that does not contain added ammonia.

2. Some histopathological effects are temporary during continuous exposure of fish to ammonia.

3. Some histopathological effects have occurred at concentrations of ammonia that did not adversely affect survival, growth, or reproduction during the same exposures.

Because of the lack of a clear connection between histopathological effects and effects on populations, histopathological endpoints are not used in the derivation of the new criterion, but the possibility of a connection should be the subject of further research.

As discussed in greater detail below, chemicals such as EDCs have been shown to produce a wide variety of measurable changes at many different levels of biological organization. The challenge is to select from among those the endpoints that have sufficiently clear connection to expected effects on populations of aquatic organisms.

3.2.4.1 Specific Examples of Measurable Changes at Different Levels of Biological Organization

The range of organismal endpoints that have been reported in the literature is vast, and varies to some degree on the organism and toxicant. With respect to only the HPG axis in vertebrates, this range of endpoints over and above direct measures of survival, growth, and reproduction includes:

- Biochemical measures (e.g., the female-specific yolk precursor protein vitellogenin; native hormones estradiol, testosterone, 11-ketotestosterone);
- Histopathological measures (e.g., proportion of spermatogonia, presence of testis-ova, oocyte atresia, Leydig cell hyperplasia/hypertrophy);

- Gross morphology (e.g., secondary sex characteristics: nuptial tubercles, coloration, ovipositors); and
- Behavioral measures (e.g., nest building, defense/aggression).

A comprehensive survey and evaluation of all such endpoints is far beyond the scope of this document. In lieu of that, this section presents in depth discussion of several individual measures relevant to the HPG axis, including their strengths and weaknesses as direct indicators of likely population level effects. The point of this discussion is simply to provide examples of the issues that must be considered in making a decision as to the biological importance and scientific defensibility for a specific endpoint, organism, or toxicant as it pertains to ALC derivation. These decisions will likely require case by case consideration; in certain circumstances, the suitability of a particular endpoint may vary across chemicals depending on how an individual chemical influences that endpoint.

One of the challenges that arises when incorporating alternative endpoints into criteria derivation is the need to not only conclude that the endpoint warrants consideration, but also establish some definition of what level of effect on that endpoint is unacceptable. While these links may not be completely quantitative, one would not want the definition of an unacceptable effect on one endpoint to be grossly disproportional to that considered unacceptable for another (i.e., if a 20% reduction in reproduction is considered unacceptable, what degree of estradiol (E2) suppression is equivalent to a 20% reduction in reproduction?).

In the text that follows, endpoints are categorized as being either "organizational" or "activational." Organizational endpoints are those that are a result of changes to the normal growth and development of an organism, and are generally not reversible with cessation of exposure. Activational endpoints are those that occur in comparatively plastic tissues in response to exposure, but which may revert to their prior or normal condition with cessation of exposure.

Organizational Endpoint: Sex Reversal

Exposure of developing fish to endocrine-active materials during sensitive "windows" in early development can skew phenotypic sex dramatically toward either females (estrogenic chemicals) or males (androgenic chemicals). This response has been exploited by aquaculturists, who for many years have used potent natural or synthetic steroids to produce mono-sex stocks. The sensitivity of fish to this type of "sex reversal" is species-specific, and critical windows of exposure can vary markedly across species. The response can be manifested in several different ways, ranging from more or less completely sex-reversed animals (i.e., occurrence of gonads and secondary sex characteristics completely reflective of the opposite sex) to more subtle changes, such as the occurrence of intersex gonads (discussed further below). A significant challenge in assessing this condition-either in the lab or field-is knowledge of actual genetic sex of the fish. Since the molecular basis of sex determination in many fish is not known, reliable genetic markers of what sex an animal is programmed to be are not available for most test species (one notable exception here is the Japanese medaka, which is commonly used for endocrine testing in some parts of the world; Ankley and Johnson 2004). The net result of this is that the only way to practically monitor sex reversal in most fish species is indirectly, through analysis of sex ratios (generally based on phenotypic sex). This requires, of course, considerable background

knowledge concerning "normal" sex ratios for a species (or even strain) of fish. For some lab test species (e.g., fathead minnow), the normal sex ratio appears to be about 1:1, while for other commonly-tested small fish models (e.g., zebrafish), the ratio can be quite variable (Ankley and Johnson 2004). In field studies, collection of accurate sex ratio data also can be exceedingly difficult, depending on variables such as sampling gear and location and timing of collections.

Changes in the sex ratio of populations of fish, either in the lab or field, can be quite indicative of an endocrine-specific MOA, indicating exposure to estrogenic or androgenic chemicals (or even chemicals that block the actions of sex steroids). Significantly, from a risk assessment perspective, alterations in sex ratio could also have direct implications for spawning success and population viability. The degree to which sex ratios are critical in determining embryo production will vary based on reproductive strategies of the species of concern (e.g., broadcast versus paired spawners); however, from an evolutionary perspective, one would speculate that any departure from normal sex ratios for a species/population might be considered maladaptive.

Organizational Endpoint: Intersex

Exposure to certain classes of endocrine-active chemicals during critical windows in early development can produce intersex gonads (commonly termed testis-ova), a situation in which the gonads simultaneously contain both ovarian and testicular tissue. Different studies from around the world have shown an elevated occurrence of intersex fish downstream of municipal effluents containing natural and synthetic steroidal estrogens, including EE2 (WHO 2002). In fact, collection of intersex fish from the field has been one of the most visible manifestations of the effects of EDCs on fish/wildlife. For example, in a widely publicized USGS study, Blazer et al. (2007) recently reported that in the South Branch of the Potomac River and select nearby drainages, more than 80 percent of all the male smallmouth bass sampled had oocytes growing within their testicular tissue. Although histology is required to determine and quantify intersex, the techniques involved are relatively straight-forward. What is more challenging than measurement is interpretation of the results. For example, it appears that some degree of background intersex can occur, even in species held under carefully-controlled conditions (Grim et al. 2007). The degree of background intersex and sensitivity to chemically-induced intersex appear to be quite species-specific, requiring a thorough understanding of normal gonad differentiation and development in the species of concern.

Even in species for which background intersex is low, there is uncertainty as to the degree to which the condition could occur and not interfere with normal reproductive function. For example, in a field study in the UK, Jobling et al. (1998) noted a wide range of intersex in roach collected, even from the same site, with severity of the response ranging from occurrence of a few primary oocytes in otherwise normal testicular tissue to instances where there was a complete absence of sperm ducts in the males. Arguably, the former fish could produce viable sperm, while the latter certainly would not. So, although intersex is an intuitively reasonable endpoint upon which to base predictions of possible adverse effects of endocrine-active chemicals on reproductive success, determination of the relationship between severity of the condition and production of viable embryos is required to conduct this analysis.

Activational Endpoint: Behavior

Although not usually considered a biomarker in the traditional sense, behavior is an endpoint that historically has been seldom used for ecological risk assessment, including the derivation of ALC. There are several reasons for this: (1) the types of assays used to assess behavior can be quite labor-intensive, (2) many methods for assessing toxicant-induced behavior have some degree of subjectivity, (3) many behavioral changes (e.g., gill ventilation in fish) are relatively non-specific in that they do not necessarily reflect exposure to chemicals with a specific MOA, and (4) translation of behavioral changes into adverse effects on endpoints such as survival, growth and reproduction can be difficult. Nonetheless, virtually all environmental toxicologists recognize the potential for chemically-induced alterations in behavior to influence the health of individual animals and populations.

There are some compelling reasons to consider behavior as a potentially useful/important endpoint in assessing the ecological risk of certain classes of endocrine-active chemicals. First, estrogens and androgens are known to play relatively specific roles in a variety of reproductive behaviors in fish, including competition for mates, courtship and nest-holding/guarding. Alterations in any of these behaviors theoretically could affect reproductive success and, hence, population status. In recognition of this there have been several recent papers describing straight-forward, relatively quantitative assays for assessing the effects of endocrine-active substances on fish reproductive behavior. For example, Martinovic et al. (2007) conducted a study in which they showed that male fathead minnows exposed to a relatively low concentration of 17β-estradiol, and subsequently placed in a competitive spawning situation with non-exposed males, failed to compete successfully for nesting sites/females. Similar types of results have been reported for other fish species exposed to steroidal estrogens (e.g., EE2; see Part II of this white paper), suggesting that behavioral alterations could be important to consider, especially if they occur at exposure concentrations below those that cause effects on more traditional endpoints such as development and egg production.

Activational Endpoint: Secondary Sex Characteristics

As described above, exposure of developing animals to endocrine-active chemicals can alter phenotypic sex, resulting in skewed sex ratios in populations. These organizational changes observed in secondary sex characteristics (in sexually dimorphic species) and/or gonads typically are not reversible. However, it also is possible to alter secondary sex characteristics, usually in a reversible manner, in sexually-mature fish through exposure to endocrine-active substances. For example, estrogens or anti-androgens can reduce expression of androgen-dependent secondary sex characteristics in males. Similarly, androgenic chemicals can cause female fish to develop male secondary sex characteristics, such as nuptial tubercles in the fathead minnow or elongated anal fins in the Japanese medaka (Seki et al. 2006). Alterations in secondary sex characteristics are much less useful indicators of endocrine-mediated responses in test species, such as zebrafish, with limited sexual dimorphism (Seki et al. 2006).

Alterations in secondary sex characteristics in adult fish can, in some instances, be subtle and somewhat subjective with respect to interpretation. For example, reductions in the status of

existing structures in fish (such as nuptial tubercles in male fathead minnows or anal fin length in male medaka) can be difficult to quantify. However, when there is a *de novo* synthesis of structural characteristics where none previously existed (such as tubercles in female fathead minnows), the response is not only quite specific (in this case to an androgenic MOA), but very easy to detect (i.e., the baseline, control condition is zero).

Although changes in secondary sex characteristics appear to be reasonable mechanistic biomarkers for some endocrine MOA, their utility as a predictor of adverse outcomes (e.g., egg production) is uncertain. Specifically, given our current understanding of fish reproductive physiology/endocrinology, causative links between secondary sex characteristics and gamete quality would be difficult to define. At best, a correlative association may be identified between the two parameters. For example, in studies with the synthetic androgenic steroid 17β-trenbolone, egg production appeared to be reduced at about the same test concentration that caused some degree of nuptial tubercle formation in females (Ankley et al. 2003).

Activational Endpoint: Vitellogenin

Vitellogenin status is probably the most commonly measured endpoint in studies with endocrine-active chemicals in fish. Measurement of the lipoprotein (or its mRNA) is relatively easy via a variety of methods (although most techniques have some degree of species specificity; Wheeler et al. 2005). Production of mRNA (*vtg*) and protein (VTG) in the liver of female oviparous (egg-laying) vertebrates is normally stimulated by activation of the estrogen receptor by endogenous estradiol. The protein is released to the plasma and subsequently deposited in the ovary where it forms a key constituent of developing oocytes. VTG levels in male oviparous animals typically are non-detectable due largely to very low circulating estradiol concentrations; however, males retain the molecular "machinery" in the liver necessary to produce VTG. Hence, exposure to even relatively low amounts of exogenous estrogen or estrogen mimics can stimulate a marked induction of VTG in males. The response not only is specific and sensitive (in part due to a baseline of essentially zero), but relatively sustained after exposure, as the males have no mechanism whereby to clear the protein from their blood.

Although *vtg* and/or VTG induction in male fish is an excellent biomarker of exposure to estrogens (Lattier et al. 2002), the response appears to have little direct (i.e., causative) value in terms of predicting adverse effects on reproduction (e.g., Wheeler et al. 2005). This perhaps should not be surprising given that VTG production in males is not part of any normal physiological pathway. It is possible, however, that correlative relationships between *vtg* and VTG induction in males by exogenous estrogens (such as EE2) and overall effects on fish population status could be derived (e.g., Kidd et al. 2007). This certainly merits additional study, but at present, it appears that the most technically-defensible use of VTG occurrence in male fish is as an indicator of exposure to estrogenic substances in the field and/or confirmation of chemical MOA in laboratory studies.

As opposed to males, VTG has a clear physiological role in females in that the protein is essential to egg production. Concentrations of VTG in females can be reduced by endocrine-active chemicals that directly or indirectly inhibit steroid (ultimately estradiol) production. For example, aromatase inhibitors such as some conazole fungicides decrease steroid production by

inhibiting enzymes involved in steroidogenesis, while other androgenic chemicals like trenbolone decrease steroid production through feedback inhibition in the HPG axis. As a consequence, these classes of endocrine-active chemicals reduce normal VTG production in female fish, thereby reducing fecundity and, ultimately, affecting population status (Miller et al. 2007). Therefore, in the case of females, VTG status may be effectively used as a biomarker both of exposure and effects. Kidd et al. (2007) found that VTG was elevated in female fathead minnows outside of their spawning season. Therefore, elevated VTG in females outside of the spawning season may also be an important measure of stress.

3.3 Pathways and Receptors Beyond the HPG-Axis

As was explained at the outset, this section (Section 3) has a substantial focus on the HPG-axis not because it is the only MOA that is of concern in this document, but because it is currently prominent in both social and scientific arenas. However, it is important to re-emphasize that the use of HPG-active chemicals as a basis for discussion does not imply that this is the only group of CECs of concern with respect to the development of ALC, or the only group for which there may be need for supplementation of the explicit procedures outlined in the *Guidelines*.

As an example, the hypothalamic-pituitary-thyroid (HPT) axis is another endocrine system present in vertebrates that governs important biological pathways and is potentially subject to disruption. Similar to the role of steroid hormones in the HPG axis, actions of the HPT axis are mediated through thyroid hormone, which is involved in the regulation of metabolic activity, energy consumption and muscular activity in adult animals, and the regulation of postembryonic or perinatal growth and development in developing animals, especially in the central nervous system (Chatterjee and Tata 1992). Thyroid hormone is also responsible for the obligatory induction and maintenance of metamorphosis in amphibian and other poikilotherms, and may also play a role in male reproduction (Peterson et al. 1997). Since the actions of thyroid hormone are mediated via binding to highly-conserved nuclear thyroid hormone receptors and modulating transcription of specific genes, disruption of the HPT axis can be disrupted in many ways parallel to those discussed for the HPG axis (Farwell and Braverman 2006), and in doing so, create similar challenges for the development of ALC. Only a few of the developmental actions of thyroid hormones, however, are the result of the direct interaction of the hormone and receptor. Instead, most are indirect via the influence of thyroid hormone on other hormone or growth factors. For example, some of the growth-promoting effects of thyroid hormones on juveniles are indirectly mediated via growth hormone released from the pituitary gland (Chatterjee and Tata 1992).

The amphibian metamorphosis assay is one of the thyroid-relevant *in vivo* screening assays EPA has developed to detect chemicals that interfere with the thyroid hormone system. The assay represents a generalized vertebrate model to the extent that it is based on the conserved structure and functions of the thyroid systems, and thus mirrors some of the assays developed and discussed earlier for the HPG axis. This particular assay is important because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to substances active within the HPT axis (Fort et al. 2007). The utility of this and other similar HPT-specific assays for development of ALC is predicated on the principle that the dramatic morphological

changes that occur during post-embryonic development of vertebrates are dependent on the normal function of the HPT axis, and that interference with this process leads to quantifiable effects (Zoeller and Tan 2007).

Other pathways relevant to this discussion could include any of a number of those regulated by different nuclear hormone-type transcription factors, such as the progesterone, glucocorticoid and aryl hydrocarbon (Ah) receptors. Of these the Ah receptor is of particular interest because it has been well studied and is key to the toxicity of several important environmental contaminants such as dioxins and PCBs. Ah receptor agonists are extremely toxic to early life stages of some vertebrates species (e.g., adult fish are at least 10 times less sensitive than early life stages), can induce delayed mortality not captured in short-term (e.g., 96-hour) toxicity tests, and are not very toxic to invertebrates, which lack the receptor (Cook et al. 1993; Mount et al. 2003; Tanguay et al. 2005). Hence, as is true for HPG-active chemicals, knowledge that a contaminant may be an Ah receptor agonist can help focus testing to determine ecological risk (Cook et al. 1993).

Although the previous systems are generally found in vertebrates but not invertebrates, parallel developmental, reproductive, and homeostatic systems exist in invertebrates (Lintelmann et al. 2003) and are most likely just as susceptible to disruption by xenobiotic chemicals. In fact, many pesticides are designed explicitly to disrupt biochemical pathways specific to invertebrates or sub-groups of invertebrates as a means to reduce effects on non-target (vertebrate) organisms. Some endocrine-mediated processes unique to certain taxa of invertebrates include molting, limb generation, diapause, pheromone production, pigmentation and coloration, and metamorphosis. For these processes, the most important endocrine regulators in arthropods are ecdysone and related compounds (ecdysteroids), which are involved in embryonic development, molting, metomorphosis, reproduction, and pigmentation (Lintelmann et al. 2003). Juvenile hormones in insects and methylfarnesoate in crustaceans (both belonging to the class of sexual hormones called terpenoids) are also deemed necessary to mediate the regulatory functions of ecdysteroids (DeFur et al. 1999). Research on the effects of CECs on these systems is still in its early stages, but the parallels with other systems that are susceptible to disruption are clear, and may therefore create similar issues for the development of ALC.

4.0 SUMMARY AND RECOMMENDATIONS

Through its deliberations, the workgroup concluded that the basic framework and conceptual underpinnings of the *Guidelines* apply to CECs as well as other chemicals. Further, the "Good Science" clause of the *Guidelines* provides the flexibility to adopt procedures that will produce a technically rigorous and protective criterion. The focus of this report has been the interpretation and adaptation of the principles set forth in the *Guidelines* with respect to common toxicological characteristics of CECs. In that regard, the workgroup identified a number of possible modifications or alternate interpretations that might aid those developing criteria for CECs to do so in a resource efficient manner that takes best advantage of existing knowledge.

Although some of the recommendations involve increasing flexibility in meeting certain data requirements, the intent is to guide the generation of ALC for CECs that have the same technical rigor as 304(a) criteria developed for other chemicals; these are not methods for "short-cut" criteria. This is a significant point, because an important feature of the *Guidelines* is defining a minimum technical rigor that criteria must have; if insufficient information exists to achieve a minimum level of confidence in the calculated criterion, then criteria should not be derived. The important consequence of this for risk assessors and managers is that when criteria are used to make regulatory decisions, one can have confidence that uncertainty regarding the criterion is not excessive. In other words, criteria derived using the *Guidelines* are often used as both "walk away values" (i.e., there is high confidence that there is little or no risk when exposures are below criteria) and as indicators of risk (implying that effects are likely when criteria are exceeded). If greater uncertainty were allowed in criteria, then the ability to use the values in this way would be compromised.

A negative aspect to establishing a minimum level of information for criteria is that there may be chemicals for which regulatory guidance is needed, but for which toxicological data are insufficient to meet the minimum standards of the *Guidelines*. In such cases, there may still be a need for alternate approaches to derive interim regulatory guidance values on which to base decisions that must be made before sufficient information for a complete water quality criterion can be gathered. While much of the discussion in this report might be useful to inform the development of such an approach, it must be emphasized that developing procedures to derive interim regulatory guidance values based on limited toxicity information is a separate matter and would require considerable additional analysis.

The subsequent sections summarize the issues and recommendations of the workgroup according to the areas of concern identified above.

4.1 Relevance of Acute Toxicity Effect Levels in Setting ALC for CECs

Some CECs may not be acutely toxic, or may only be acutely toxic at environmentally irrelevant concentrations. Thus, if the minimum data requirements for acute toxicity data are not already met by existing data, conducting additional acute tests might be unwarranted. Indication of lack

of acute toxicity in key aquatic species might also warrant direct calculation of the CCC rather than using the FAV/FACR approach, and thus eliminate the need for the full suite of acute toxicity tests normally required.

For a CEC of interest, available information should be reviewed to determine if the CMC would be sufficiently higher than the CCC such that developing the CMC is not needed. Exactly how much data is a risk management judgment, and probably does not have a unique answer. We recommend that the following information be considered when addressing this issue:

- the amount and phylogenetic spread of acute toxicity data available;
- toxicity data from short-term exposures that may not meet the strict definitions in the *Guidelines* of acute toxicity data acceptable for criteria derivation, but from which information on responses to acute exposures can be inferred;
- data on short-term exposures garnered from longer-term exposures;
- information from closely related chemicals thought to have the same MOA that have more robust acute data sets; and
- knowledge of the degree of phylogenetic distribution of the toxicity pathway of concern.

4.2 Defining Minimum Data Requirements in Terms of Taxonomic Coverage

One consequence of dropping acute testing requirements in criteria derivation is the inability to calculate a CCC using the ACR approach, i.e., as the quotient of the FAV and FACR. In addition, for chemicals with large ACRs, it is likely that the mechanisms for acute and chronic toxicity differ (Welshons et al. 2003) and that the sensitivity of different taxa to acute and/or chronic toxicity varies widely. Both of these issues introduce uncertainty into the interpretation of ACRs, and probably make it inadvisable to use the FAV/FACR approach. Under such a circumstance, a prudent approach would generally be to develop a CCC directly from a sufficiently robust set of chronic data, using the procedures outlined in the *Guidelines*. If there is insufficient data from actual toxicity tests to fulfill the MDRs to develop a CCC directly from chronic toxicity data, a reasonable understanding of the toxicological MOA for the chemical may allow inferences as to what taxa (and endpoints) are most likely to be insensitive, such that measured chronic values for those taxa might not be needed. One important consideration in this process is to avoid an excessive number of taxa estimated to be insensitive, relative to those for which actual test results are available, and thus to distort the phylogenetic distribution from that implicit in the MDRs and typical of ALC.

Accordingly, the workgroup recommends that, for chemicals without complete chronic toxicity data sets fulfilling all MDRs, there be an evaluation of whether sufficient information exists to conclude that certain taxa would not be sensitive to the chemical. Given the variation in understanding and availability of data likely to exist for different CECs, it is presumed that at least initial application of this approach would have to be justified on a chemical-by-chemical basis using appropriate scientific judgment. However, lines of evidence that might be applicable to this determination include:

- an in depth understanding of the toxicological (or, in the case of drugs, therapeutic)

MOA;

- information on the basic physiology of other taxa in relation to the MOA;
- toxicity data from chronic exposures or other relevant experiments that do not meet the strict definitions of acceptable chronic data given in the *Guidelines*, but from which information on relative taxon sensitivity can be inferred; and
- information from closely related chemicals thought to have the same MOA that have more robust acute or chronic data sets.

4.3 Use of Non-Resident Species in ALC Development

Historically, EPA has not used data derived from toxicity testing with non-resident species in the actual criteria derivation process. Excluding species simply because they are not resident may be unnecessarily restrictive for the purposes of deriving national criteria, and may actually increase rather than decrease uncertainty. Because ALC are intended to protect "most of the species, most of the time" and use distributions of test data for point estimation, increasing the species representation in the toxicological database should allow better estimation of species sensitivity distributions.

The workgroup recommends that some non-resident species be considered for use in criteria derivation calculations, focusing on those species with widely used and standardized test methods and for which there is no reason to believe would misrepresent the sensitivity of comparable resident species. Furthermore, we specifically suggest accepting data for zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), to reflect international efforts toward data equivalency (Ankley and Johnson 2004). This recommendation pertains to the direct use of chronic toxicity data in the calculation of a CCC as is currently done for resident species. It is worth noting that even non-resident species that are not included in criteria calculations may still provide important information on MOA, sensitivity of endpoints, etc., as expanded on further below.

4.4 Defining Appropriate Chronic Toxicity Data

The *Guidelines* state that acceptable chronic tests for criteria derivation are full life-cycle exposures (egg/birth to egg/birth) for both vertebrates and invertebrates, as well as partial life-cycle (adult to juvenile) and early life-stage (ELS; egg to juvenile) tests for fish. For chemicals for which sexual development/maturation or reproductive effects are of most concern, the allowance in the *Guidelines* for using ELS or partial life-cycle fish tests might need reconsideration. The *Guidelines* already give priority to life-cycle tests when they are available and show greater sensitivity than other tests. However, other information indicating the importance of sexual development and reproduction (from other species, similar chemicals, knowledge of the MOA) might also establish a basis for not considering ELS data and for requiring life-cycle or partial life-cycle tests for fish.

At present, a CCC could be derived for a chemical for which chronic toxicity data for fish are limited to ELS exposures. Because of the importance of sexual maturation and reproduction for

determining the chronic toxicity of chemicals like EDCs, the workgroup recommends strengthening the *Guidelines* such that the chronic toxicity data requirements require that either:

1) Full life-cycle data be available for at least one fish species; or

2) There is a body of experimental information indicating that life processes outside the ELS or partial life-cycle exposure/observation windows would not be important to capturing the important toxicological effects of the chemical.

We note further that although this report is focused on CECs, this recommendation may be important to implement for all chemicals, not just CECs.

Regarding the latter, we recognize that there may be circumstances where the information that shows the sensitivity of different life stages comes from studies that, while scientifically valid, for some reason do not meet all the requirements of a valid life-cycle test as defined in the *Guidelines*. Alternatively, there may be data from experiments that violate other requirements of acceptable toxicity tests under the *Guidelines*, but still provide insight into sensitive exposure periods or life processes. Even though chronic values from such data may not be used directly to calculate a CCC, it seems a reasonable use of such data to evaluate the question of where in the life-cycle there are important windows of exposure and/or effect, and how that impinges on criteria derivation.

It may also be that meaningful chronic toxicity data could be developed from exposures that have a structure different from the life-cycle, partial life-cycle, and ELS protocols defined explicitly in the *Guidelines*; e.g., a short-term (21-day) reproduction assay with the fathead minnow (U.S. EPA 2001) or a multi-generational study – see example for EE2 reported in Nash et al. (2004). While defining such alternate protocols is beyond the scope of this document (see Ankley and Johnson 2004 for more detail), we recognize the potential for such a situation and leave it to appropriate implementation of the "Good Science" clause to allow for inclusion of such alternative test protocols as surrogates for chronic toxicity data, most likely in addition to, rather than instead of, data from life-cycle toxicity tests.

4.5 Selection of Effect(s) Endpoints Upon Which to Base ALC

Although chronic criteria typically are based on direct effects on reproduction, growth, and survival, there may be other endpoints indirectly related to these responses that could be useful for criteria derivation. The selection of endpoints appropriate to the derivation of ALC must be tied to the narrative intent of the overall *Guidelines*. The stated goal of ALC is to "protect aquatic organisms and their uses." While the exact meaning of "protection" is not defined, there is considerable discussion in the *Guidelines* document that makes clear that protection does not mean the prevention of any measurable biological effect in any organism. Instead, there is discussion of endpoints that are "biologically important" and prevention of "unacceptable effects"; this implies that in the context of criteria there are effects that are "biologically unimportant" and/or levels of effect that are "acceptable."

Chronic test data and other data should be examined to determine whether, for the specific chemical or MOA, endpoints beyond those traditionally used for criteria derivation may have intrinsic "biological importance" and therefore could be used as a basis for defining threshold of effect (e.g., sex ratio). Specifically, in the context of EDCs:

- Other "endocrine-sensitive endpoints" (e.g., VTG, testis-ova) should be examined to determine whether they can be relied upon as definitive indicators of other biologically important endpoints (e.g., reproduction), with the idea that they may be incorporated into calculation of the criterion. Important sources of this information would include full life-cycle tests in which these other endpoints were measured alongside traditional chronic endpoints, and may include tests with other chemicals with the same MOA (e.g., E2 for EE2).
- If endpoints, such as VTG or testis-ova, are used as direct or indirect indicators of effect, it is critically important that the baseline condition (e.g., variation during normal development) be understood sufficiently to define when changes are biologically meaningful.
- Selection of appropriate endpoints (and their associated effect thresholds) may, in some instances, transcend "biological importance" (the focus of the *Guidelines*) to reflect societal concerns (e.g., physical appearance of wild-caught fish).

4.6 Involvement of an Expert Panel

As becomes clear from the preceding issues, development of appropriate criteria for CECs may be unusually dependent on technical interpretations of a wide range of toxicological information pertinent to specific chemicals. One of the recommendations from a SETAC Pellston workshop (Mount et al. 2003), consistent with much of the above, was that expert panels be used to provide professional judgment during stages of the problem formulation and data interpretation associated with criteria development, particularly for chemicals with specific MOA. The involvement of the panel would "ensure consideration of other existing data for the chemical of concern, enable a significant degree of up-front technical input, and provide a level of peer review that should facilitate wider and more ready acceptance of the recommended criteria." The workgroup agrees with this recommendation and suggests that it be incorporated into criteria development of CECs.

To maximize effectiveness, this panel should be convened very early in the criteria development process such that it will be able to assist in problem formulation, identification of important data, and scoping of particular issues that will be important. We envision these panels as being formed around specific chemicals, or groups of chemicals with a similar MOA, in order to access the most specialized expertise available.

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WHITE PAPER

AQUATIC LIFE CRITERIA FOR CONTAMINANTS OF EMERGING CONCERN

PART II

ILLUSTRATION OF RECOMMENDATIONS USING DATA FOR 17α-ETHYNYLESTRADIOL (EE2)

Prepared by the
OW/ORD Emerging Contaminants Workgroup

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DRAFT DOCUMENT

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1.0 INTRODUCTION

In Part I of this white paper, toxicological characteristics of some contaminants of emerging concern (CECs) important to the derivation of ambient water quality criteria for aquatic life (aquatic life criteria, ALC) were described, and recommendations were made to facilitate ALC derivation for these chemicals. In Part II of this white paper, toxicity data for a model CEC, 17α-ethynylestradiol (EE2), are used to further illustrate and explore those recommendations. Ethynylestradiol was chosen as a model compound for a several reasons. First, it possesses many of the toxicological characteristics described in Part I, and sufficient toxicity data exist to allow evaluation of the principles underlying the Part I recommendations. Second, toxicological effects of EE2 have been found both in the laboratory, the source of toxicological data for criteria development, and in the field, where criteria are used to enforce the regulatory authorities of the Clean Water Act. Finally, there is interest in deriving an EE2 ALC, and using EE2 as a basis for discussion should help advance that goal. While acknowledging that interest, it is important to note that the data and discussion presented are not intended to represent the formulation of an actual ALC, and potential ALC concentrations should not be inferred. The information from the ecotoxicological literature used here is for illustrative purposes and should not be considered as comprehensive, nor have all the data been fully examined for quality and applicability to ALC development.

The synthetic estrogenic steroid EE2 is the active pharmacological component of most oral contraceptives, and acts as a potent estrogen receptor agonist in vertebrates. After use and excretion of the contraceptive, domestic sewage treatment plant (STP) effluents become the primary source of EE2 entering the aquatic environment (Damstra 2002). Kolpin et al. (2002) found EE2 in 5.7% of 139 streams monitored in the U.S. While the concentrations of EE2 in Kolpin et al. (2002) have been debated (Ericson et al. 2002; Till 2003), other studies have noted concentrations ranging from 0.1 – 5.1 ng/L in surface waters (as reviewed by Campbell et al. 2006). Overall, it is somewhat uncertain at this time how high environmental concentrations of EE2 may be. Reliable analytical methods for the detection of EE2 have not been in existence very long, nor have they been widely validated in independent multi-laboratory studies. Some modeling efforts by the pharmaceutical industry indicate that based on the level of production and use of EE2 in the U.S., concentrations found in effluents should be less than 1 ng/L (Anderson, P.D. and D'Aco, V, personal communication, 2008). Complicating assessment of the possible risk of EE2 is the fact it co-occurs in STP effluents with the natural steroid hormones estradiol and estrone, though EE2 is generally found at lower concentrations. These three estrogens reportedly account for the majority of the estrogenic activity present in domestic wastewater effluents (Desbrow et al. 1998; Snyder et al. 2001), but EE2 is the most potent and resistant to degradation of the three (Nash et al. 2004; Gross-Sorokin et al. 2006). Data collected from fish and surface waters downstream of STPs over the past decade have implicated steroidal estrogens as the primary constituents in domestic effluents leading to the occurrence of intersex fish (Gross-Sorokin et al. 2006).

The remainder of this part of the white paper consists of a brief description of some relevant acute and chronic toxicity data available for EE2 (Section 2) and the evaluation of these data with respect to the recommendations made in Part I (Section 3).

2.0 DESCRIPTION OF THE ACUTE AND CHRONIC TOXICITY DATA SUMMARIZED FOR EE2

Acute and chronic toxicity data were identified via a literature search and review of relevant articles from EPA's ECOTOX database in April 2007. This list of potentially useful articles was supplemented with a few additional reports and articles as they became published or available. Only those studies with EE2 effect data on individual aquatic organisms or their populations were retained. For this particular effort, all endpoints expressing effects of EE2 at the whole animal and cellular levels were initially considered. Because the EE2 dataset is relatively large, and because many studies report more than one endpoint of possible consideration for ALC development, the data have been broadly summarized in an appendix (Appendix A). The tables comprising Appendix A are organized by endpoint, and include separate tables for endpoints typically used to derive ALC (survival, growth and reproduction) as well as for other endpoints relevant to the estrogenic mode of action of EE2. Table A.1 contains the data available on the acute (lethal) toxicity of EE2 to aquatic animals. This table is followed by others containing the chronic (long-term) effects of EE2 on survival (Table A.2) and growth of aquatic animals (Table A.3). Tables A.4 through A.9 present data directly (fecundity, fertility) or indirectly (sex reversal, intersex, sexual behavior, vitellogenin) related to the effects of EE2 on reproduction. Finally, Table A.10 presents a summary of the significant effects of EE2 on aquatic animals based on other potentially relevant endpoints. *In vitro* effects were not considered in the data analysis.

Within each table in Appendix A, data are first separated by studies where significant effects were observed, and then by studies where significant effects were not observed (i.e., where no effect was observed at the highest concentration tested). Each table in the appendix combines data for aquatic vertebrates and invertebrates for both freshwater and saltwater species, the latter designated by asterisks. All tables are organized by increasing effect concentrations, and all chronic effect endpoints are as reported by the authors.

Many studies of the effects of EE2 on aquatic organisms did not use standard toxicity test protocols, particularly those measuring sublethal responses. This is probably due in part to these studies having been designed for purposes other than ALC development, such as exploration of toxicity mechanisms, identification of sensitivity windows, bioassay development, etc. Adequate quantification of effect concentrations is also difficult for some of these studies because of the use of widely spaced treatment concentrations and by problems with analytical detection of exposure concentrations near the threshold for reproductive effects. While the results from such studies might limit their use in ALC development according to the definitions in the *Guidelines*, they were included in this document because they may inform other aspects of criteria derivation, as explained in general terms in Part I and in detail in the sections that follow.

3.0 EE2 DATA EVALUATION AND CONSIDERATIONS FOR ALC DEVELOPMENT

This section considers the application of the data in Appendix A toward criteria derivation in the context of the several areas of concern and general recommendations identified in Part I of this white paper.

3.1 RELEVANCE OF ACUTE TOXICITY EFFECT LEVELS IN SETTING ALC FOR EDCS

One of the recommendations from Part I of this document was to determine whether the acute sensitivities of aquatic organisms to a chemical of interest are sufficient, relative to chronic sensitivity and expected exposures, to warrant derivation of a criterion maximum concentration (CMC) under the *Guidelines* procedures. This is especially important if there is not sufficient acute toxicity data to meet the minimum data requirements of the *Guidelines*, in order to avoid wasting resources on unnecessary additional testing. EE2 provides a good example of a chemical having insufficient acute toxicity data to derive a CMC according to *Guidelines* procedures, but enough data to demonstrate that deriving a CMC is not necessary.

Table 3.1 provides information on GMAVs that might be considered in CMC derivation. These values were derived from Table A.1 for any tests meeting *Guidelines* requirements, including "greater than" values indicative of the highest tested concentration eliciting less than 50% mortality. For genera without such acceptable tests, EC50/LC50s from Table A.1 for tests of 24 h duration and from Table A.2 for tests up to 30 days were also used. The EC50/LC50 values for these longer tests are designated as "greater than" values to indicate the expectation that acute EC50/LC50s would be higher. Values for medaka and zebrafish are included in accordance with the recommendation from Part I of this document that some latitude be adopted regarding species not resident to North America. Acute tests with embryos, not usually included in CMC calculations, are also included here because they suggest greater sensitivity of this life stage.

Table 3.1. Potential GMAVs for Application to EE2 CMC.

Genus	GMAV (ng/L)	Comments
Freshwater		
Rana	>760,000	14-d test
Gammarus	>840,000	10-d test
Medaka	>1,000,000	
Danio	1,700,000	
Ceriodaphnia	1,800,000	
Hydra	3,800,000	
Sida	>4,100,000	24-h test

Genus	GMAV (ng/L)	Comments
Daphnia	>5,000,000	
Chironomus	9,100,000	24-h test
Saltwater		
Lytechinus	30,000	Embryo
Strongylocentrotus	30,000	Embryo
Acartia	88,000	Embryo
Tisbe	>100,000	21-d test
Acartia	1,100,000	
Neomysis	1,200,000	

The data summarized in Table 3.1 show several deficiencies in meeting the minimum data requirements for deriving a CMC under the Guidelines. For freshwater application, only four genera, rather than the minimum of eight, meet the acute test requirements, even if the prohibition for non-resident species is ignored. If the shorter and longer tests are included, the requirement of at least eight genera is met, but the requirement for a salmonid fish is not. Even if this requirement is also ignored, two of the lowest four genera are "greater than" values, whereas CMC calculations can only be made if the four most sensitive genera have definite values ("greater than" values are permitted only for more tolerant genera.) For saltwater, there are even greater deficiencies in meeting the minimum data requirements.

Although these data are insufficient for deriving CMCs, they do provide ample evidence that a CMC is not needed and that it is unnecessary to conduct further tests to meet the minimum data requirements. For freshwater, there is still a rather broad taxonomic representation, including three vertebrates from two different classes, four crustaceans from two orders, and a third phylum. The acute LC50s/EC50s are consistently near and above 1 mg/L, several orders of magnitude above both the most sensitive chronic endpoints (Tables A.4 – A.9) and the highest environmental concentrations that organisms might be exposed to. The saltwater data do show greater sensitivity for the embryonic stages of some genera, but whether this reflects a lifestage or taxa sensitivity issue, these LC50s/EC50s are still four orders of magnitude above the most sensitive chronic endpoints and environmentally-relevant exposures.

3.2 USE OF NON-RESIDENT SPECIES IN ALC DEVELOPMENT

Under the *Guidelines*, toxicity values from aquatic species not resident to the contiguous 48 United States, Alaska, or Canada are excluded from ALC derivation. One of the recommendations in Part I of this white paper is that this prohibition be relaxed and that data for non-resident species be allowed where deemed suitable, especially for species such as medaka and zebrafish which have become standard test organisms commonly used worldwide. Any tested species, whether resident or not, serves as a surrogate for estimating a sensitivity

distribution relevant to assessing risks in a variety of aquatic communities with a multitude of untested species. Therefore, the issue here is whether a non-resident species can serve as a reasonable surrogate for assessing the sensitivity of untested resident species. The use of such species would still be contraindicated if there is reason to believe they are significantly more or less sensitive than resident species.

The data in Appendix A support the use of medaka and zebrafish data in criteria calculations. Although there are no resident fish species with which to compare the acute sensitivities of medaka and zebrafish (see Table 3.1 and Table A.1), their lack of acute sensitivity is consistent with that of resident amphibians and invertebrates in the available data. The sensitivities of these fish species for long-term survival (Table A.2), growth (Table A.3), and reproduction (Table A.4) are interspersed with those of resident fish species, so there is no indication of either substantially higher tolerance or sensitivity to contraindicate their use. This is also generally true of the other endpoints summarized in Appendix A. The similarity among fish species of different geographic origins is not surprising, since the MOA of EE2 involves receptors and pathways that are highly conserved among vertebrates. If similar trends are seen in the data once they are thoroughly examined for quality and applicability to ALC development, data from these non-resident species should be included in criteria development.

The data in Appendix A also underscore pragmatic advantages of including non-resident species in criteria development. Medaka and zebrafish provided a large fraction of the available data regarding EE2 effects on fish. Removing them from the dataset simply because they are not resident would limit information on the distribution of species sensitivity and may actually increase rather than decrease uncertainty regarding resident species. Another use of data from non-resident species could be to assist in extrapolations of information across species, chemicals, and endpoints. For example, life-cycle tests with medaka could be used to evaluate whether early life-stage or partial life-cycle tests with resident species should or should not be accepted in criteria calculations for specific classes of chemicals with a defined MOA. The relationship of reproductive effects in non-resident fish (Table A.4) to other endpoints (Tables A.5-A.9) could also be used to determine how to apply information on these other endpoints for resident species lacking direct toxicological information on reproduction.

3.3 MINIMUM DATA REQUIREMENTS REGARDING TAXONOMIC COVERAGE

As discussed in section 3.1, deriving a CMC for EE2 is not useful because acutely-toxic concentrations are so much higher than both chronic effects concentrations and expected environmental concentrations. In addition, developing a CMC would require additional acute toxicity tests to meet the minimum data requirements (MDRs) specified in the *Guidelines*. Without a CMC, the criterion continuous concentration (CCC) must be calculated directly from the available data, rather than through extrapolation using an acute to chronic ratio (ACR); this is probably not advisable anyway for such large ACRs. Since the ACR method is moot, the *Guidelines* calculation procedures for the CCC require that there be sufficient chronic toxicity tests to satisfy the MDRs for estimating the fifth percentile of the chronic database. For

freshwater criteria, these MDRs include a species from: the family Salmonidae; a species from a second family in the class Osteichthyes; a species from a third family in the phylum Chordata; a planktonic species from the Class Crustacea; a benthic species from the Class Crustacean; a species from the Class Insecta; a species from a phylum other than Chordata or Arthropoda; and a species from an order of insects or a phylum not otherwise represented.

Few existing ALC have chronic data that meet the MDRs, and this will likely be true of CECs as well. Significant expense would be incurred conducting new chronic tests to satisfy all the requirements. As recommended in Part I of this white paper, because only the four most sensitive genus mean chronic values (GMCVs) are used in the criteria calculations, chronic testing requirements for a taxon needed to meet an MDR should be waived if there is sufficient information to conclude that this taxon is more tolerant than the four most sensitive genera. A value (or values) for this taxon would still be included in the data set, but its GMCV would simply be specified to be greater than the fourth lowest GMCV.

Table 3.2 lists chronic values for the toxicity of EE2 to various freshwater genera to illustrate data that might be included in freshwater criteria calculations. These chronic values were obtained from Tables A.2-A.4, using *Guidelines* data selection procedures where possible, but also included some additional data to support discussion of how certain data deficiencies might be addressed. For invertebrates, the *Guidelines* require life-cycle tests that include reproductive endpoints, but if that type of test was not available, then other tests are reported here, with their limitations noted. For fish, the *Guidelines* preference order of life-cycle, partial life-cycle, and early life stage (ELS) was followed, but other tests were also reported as needed for illustrative purposes, with their limitations also noted. For all genera, the most sensitive endpoint among chronic survival, growth, and reproduction was selected, which was from the reproduction data of Table A.4, except for *Chironomus* (for which development from egg to pre-emergence was tested and the effects concentration was from Table A.3). Each chronic value (CV) was calculated as the geometric mean of the reported no observed and lowest observed effect concentrations (NOEC and LOEC) for an adverse effect. When the LOEC was the lowest exposure concentration, a "less-than" concentration is reported for the CV and, when the NOEC was the higher exposure concentration for insensitive species, a "greater-than" concentration is reported. As explained in Sections 1 and 2, these data are still under review and subject to modification. The specific values here should not be misconstrued as final, but rather as examples to illustrate trends and indicate needs that support the recommendations being addressed here.

Table 3.2. Potential Chronic Values for Application to EE2 CCC.

Genus	CV(s) (ng/L)	Notes
Danio	0.6, 1.5, <1.1	Life-cycle tests; for 1.5 ng/L CV, there was a 9-fold difference between LOEC and NOEC and the LOEC was a 100% effect
Pimephales	<0.32, 1.5	Life-cycle tests; for <0.32 ng/L CV, LOEC showed reduced fertilization but increased egg production so total reproduction not adversely affected; for 1.5 CV, 4-fold difference between NOEC and LOEC
Oryzias	3.2	F_0 from 1 d through spawning; 10-fold difference between NOEC and LOEC
Oncorhynchus	<16	Adult exposure only; fertilization success only endpoint examined
Potamopyrgus	50	Adult exposure only; embryo production over 9 wk test
Gammarus	>7,600	Population size over 100 d test; increased population size at 760 and 7,600 ng/L
Daphnia	45,000	5-fold difference between NOEC and LOEC
Tisbe	>100,000	Saltwater copepod included to further indicate arthropod insensitivity
Chironomus	320,000	Larval growth and molting schedule only; did not include emergence and reproduction
Brachionus	800,000	Intrinsic rate of population increase over 72 hr test

The data in Table 3.2 indicate high sensitivity of vertebrates to EE2. Significant reproductive effects in the life-cycle tests for zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) occur at concentrations near and perhaps below 1 ng/L. Although the chronic test for medaka (*Oryzias latipes*) did not cover the entire life cycle, it included life stages likely important for reproduction and indicated a sensitivity similar to zebrafish and fathead minnow. For rainbow trout (*Oncorhynchus mykiss*), a more limited exposure addressing only effects on fertilization suggests that reproductive effects on this species should also be present in the low ng/L range. However, the absence of a definite toxicity value for rainbow trout will be an important impediment to criteria calculations, both for leaving an MDR unsatisfied and for being one of the four most sensitive genera in this set. Actual criteria development will require a decision whether to (a) require more information for this species, (b) use other information to help estimate rainbow trout sensitivity or (c) justify setting the MDR aside (see Section 3.5).

The invertebrate data in Table 3.2 indicate lower sensitivity, especially for arthropods (*Gammarus, Daphnia, Tisbe, Chironomus*) and rotifers (*Brachionus*). Some data, like the *Chironomus* test, fail to satisfy the *Guidelines* requirement for a life-cycle test and the copepod *Tisbe* is a saltwater species included here only to reinforce conclusions about arthropod sensitivity. Also, the tests for *Gammarus* and *Brachionus* are not standard life-cycle tests, but could be considered to satisfy *Guidelines* requirements because exposures span a life cycle and

include reproductive effects. The snail (*Potamopyrgus*) toxicity test showed moderate sensitivity, although still about an order of magnitude less than the fish, and also does not involve a full life-cycle test.

These data demonstrate the potential for a situation in which the GMCVs for taxa reasonably expected to be insensitive do not need to be quantified. For example, although the *Chironomus* test was not a full life-cycle test and thus could not fully define the GMCV under *Guidelines* requirements, it indicates such a degree of insensitivity for growth and development, such that it can be reasonably presumed that a full life-cycle test would still show much less sensitivity than the vertebrates, especially because other arthropods are observed to be similarly insensitive. Likewise, the snail test, although not for a full life-cycle, involved the effects of long exposures on reproduction, and can be argued to be sufficiently less sensitive than fish reproduction so that it would not reasonably be expected to be among the four most sensitive genera if a life cycle test was conducted. These inclusions, along with the data for *Daphnia*, *Gammarus*, and *Brachionus*, satisfy the *Guidelines* MDRs for invertebrates, and would allow an ALC to be calculated from the four sensitive vertebrate genera, provided the value for the rainbow trout was resolved.

Assessing that taxa are likely to be insensitive could involve other lines of evidence, especially for CECs with more limited chronic toxicity data than EE2. Tests involving endpoints such as those in Tables A.5-A.10 could be used to establish that certain taxa are sufficiently less sensitive than others to preclude the need for tests on their chronic survival, growth, and reproduction (Tables A.2-A.4), the endpoints typically used in ALC development. Information from other chemicals might also be used, such as using the insensitivity of arthropods to EE2 to preclude testing this taxonomic class with chemicals with the same MOA. Such a strategy could be used to help the evaluation of EE2, particularly regarding the snail sensitivity. For example, the sensitivity of this or similar species relative to that of vertebrates for other chemicals could be used to strenghten a conclusion that they are less sensitive to EE2 than are fish.

3.4 DEFINING APPROPRIATE CHRONIC TOXICITY DATA

As discussed in Part I of this white paper, characteristics of some CECs require that careful consideration be given to the selection of chronic toxicity data appropriate for ALC development. Specifically, the use of data from early-life stage (ELS) or partial-life cycle (PLC) exposures as estimates of life-cycle chronic effect thresholds is inadvisable for chemicals whose MOA would result in biological effects for which critical periods of induction and/or expression would lie outside the exposure/observation window provided by the test procedure.

An examination of data specifically for EE2 provides evidence to support emphasis on full life-cycle exposures for determining the chronic toxicity of EE2. Länge et al. (2001) conducted a full life-cycle chronic exposure with fathead minnows which were exposed from fertilized eggs (F_0) through maturation, spawning, and early-life stage development of the F_1 generation. Nominal exposure concentrations of EE2 were 0.2, 1, 4, 16, and 64 ng/L (for convenience, nominal concentrations are used in this discussion as the important point is relative endpoint sensitivity

rather than absolute concentrations inducing effects). As part of this exposure, measurements of growth (as length) and survival were made at 28 days post hatch (dph) which would correspond to the end of a standard ELS exposure with fathead minnows. At 28 dph, there were no effects on survival. The length endpoint showed a 16% reduction at 64 ng/L, a smaller but significant reduction of 6% at 16 ng/L, and no effect at 4 ng/L. Accordingly, the NOEC and LOEC for an ELS test with EE2 would have been 4 and 16 ng/L, respectively, and an EC20 based on length would be >64 ng/L. However, as exposure continued throughout the life cycle, pronounced effects were observed for other endpoints at lower exposures. There was no reproduction at all in fish exposed to 4 ng/L, and a trend, though not significant, toward lower reproduction at 0.2 and 1 ng/L. Other significant effects observed included a 16% reduction in weight of adult female fish at 1 ng/L after 301 d exposure, and 5 to 10 percent reductions in weight of F_1 offspring at 28 dph, though the authors questioned the biological significance of the F_1 growth effects. Regardless, the clear indication is that life-cycle exposure showed substantially greater sensitivity to EE2 than was evidenced from ELS endpoints alone. This was much larger than the factor of 2 difference generally found for other chemicals by McKim et al. (1978).

A similar conclusion can be drawn from the study of Parrott and Blunt (2005). This involved exposure from fertilized egg through reproduction, including measures of fertilization success (but not ELS development) in the F_1 generation. Exposure was to nominal concentrations of 0.32, 0.96, 3.2, 9.6, and 32 ng/L EE2. Measurements of survival and growth at 30 dph showed no effects (NOEC $\geq$ 32 ng/L). However, continuation of exposure through adulthood showed no reproduction in the 3.2, 9.6 and 32 ng EE2/L treatments, and all fish in these treatments were phenotypic females. There was also suggestion of effects on fertilization success at 0.32 and 0.96 ng/L, although interpretation of these effects is complicated by an increase in number of eggs produced in these same treatments, such that the total number of fertilized eggs was not as dramatically affected. Regardless, the message relative to definition of chronic sensitivity is the same in that effects were apparent after life-cycle exposure at concentrations well below those that would be expected to show effects in an ELS test.

Additional comparisons can be extracted from the work of Wenzel et al. (2001), who conducted a multi-generational study of zebrafish exposed to EE2 concentrations from 0.05 to 10 ng EE2/L. Observations of survival and length of exposed fish showed no effects at 21 and 42 dph (NOEC $\geq$10 ng/L). However, with continued exposure, a variety of effects were observed around an EE2 test concentration of 1 ng/L, including effects on adult length, time to spawning, egg production and fertilization. As for the fathead minnow studies, survival and growth measured during the period comparable to an ELS study were far less sensitive to EE2 exposure than were endpoints measured in full life-cycle studies (Tables A.2, A.3, A.4).

The reason for these differences between ELS and full life-cycle tests is obvious when one considers the MOA for EE2, which interferes with sexual differentiation, development, maturation, and spawning. Because the endpoints measured in ELS tests are limited to survival and growth, and because the effects of EE2 on sexual differentiation are not apparent (at least not at a gross morphological level) in the tested species by 28-30 dph, the ELS test is comparatively insensitive to toxicity mediated through an estrogen receptor signaling pathway. It is interesting

to note that even though a standard ELS test is relatively insensitive to detecting the effects of EE2 exposure, other work has shown that key windows of exposure do in fact occur during the ELS exposure window. Van Aerle et al (2002) demonstrated that larval fathead minnows exposed to EE2 only during brief windows during early development (e.g., 10-15 dph) showed altered sexual development of male fish at 100 dph, including the development of an ovarian-like cavity and changes in the distribution of testicular cell types (Table A.6). The issue for interpreting chronic toxicity data is that, even though effects may be induced during ELS exposure, they are not expressed unless exposed fish are observed later in sexual development.

This latter observation also has implications for the suitability of PLC tests for detecting the effects of EE2 or other chemicals acting through a similar pathway. As discussed in the *Guidelines*, PLC tests are acceptable chronic tests for fish species that require more than one year to reach sexual maturity, such as the common species of trout. PLC tests are to begin exposure "with immature juveniles at least 2 months prior to active gonad development, continue through maturation and reproduction, and end not less than 24 days (90 days for salmonids) after the hatching of the next generation." If salmonids (or other species for which PLC tests might be conducted) were to express effects from larval exposure to EE2 as observed by Van Aerle et al. (2002) for fathead minnows, one would expect that a PLC exposure would not be as sensitive as a full life-cycle exposure. That is, even though a PLC test includes the observation periods shown to be sensitive in full life-cycle exposures, it might not include the exposure windows important to inducing chronic effects on sexual differentiation and development.

While the rationale for emphasizing full life-cycle chronic tests has clear grounding in the MOA for EE2, it has practical implications in terms of the fish species likely to be tested. Most species for which life-cycle chronic tests are most commonly conducted (primarily fathead minnow, zebrafish, medaka, flagfish, and sheepshead minnow), are small fish that develop rapidly and are continuous spawners (as opposed to annual spawners like rainbow trout or bluegill sunfish). Whether or not these life history traits influence sensitivity to EE2 is unknown, but because of the investment necessary to conduct true life-cycle exposures with annually spawning fish that take much longer to develop, it may be unlikely that comparative data will be developed. Better understanding these implications is a worthy subject for future research.

Finally, there are some scattered indications in the literature for trans-generational effects of EE2 exposure. As mentioned above, Länge et al. (2001) found small effects on growth in the F_1 generation that were not observed after comparable exposure of the F_0. Wenzel et al. (2002) also report some suggestions of growth inhibition in subsequent generations at exposure below those causing such effects in the first exposed generation (Table A.3). The mechanisms by which such effects might occur are not clear, nor are their implications (in fact, Länge et al. actively dismiss them as being biologically unimportant). At this point, it does not seem that the evidence for trans-generational effects is sufficient for requiring their inclusion in the definition of an acceptable chronic test, but the potential for the existence and importance of trans-generational effects should be re-evaluated in the future as additional data become available.

3.5 SELECTION OF EFFECT(S) ENDPOINTS UPON WHICH TO BASE ALC

Aquatic studies with EE2, particularly those using fish, have measured a variety of endpoints not traditionally used for criteria derivation, including reproductive behavior, abnormal sex ratios, changes in secondary sexual characteristics, altered histopathology (typically gonadal), changes in steroid hormones, and modifications in the expression (or activity) of a variety of proteins/enzymes. Many of these endpoints were evaluated because they are known (or hypothesized) to be responsive to estrogenic MOA, and not because the intended result was the quantitative assessment of risk. Among the challenges in using data from these types of mechanism-based endpoints is that such measurements are seldom standardized or straightforward in their interpretation. For example, alterations in behavior are difficult to objectively quantify, it is challenging to accurately measure steroid hormone concentrations in small fish, and the capability of measuring gene expression or enzyme activity can be quite lab/method-specific. A second source of uncertainty in using most of the mechanism-specific endpoints evaluated in EE2 studies is a lack of knowledge concerning the functional relationship between changes in endpoints and responses of primary concern for risk assessment, such as survival, growth and reproduction. Even in considering these challenges related to measurement and interpretation, however, there are a handful of mechanistic endpoints/responses that exhibit utility for supporting ALC derivation for EE2 (or other xenobiotic compounds with estrogenic activity). Three of these are discussed in greater detail below.

A frequently measured mechanism-specific endpoint in fish exposed to EE2, is induction of vitellogenin mRNA (*vtg*) or expression of circulating vitellogenin protein (VTG) in males (Table A.9). The most attractive attribute of this endpoint is its specificity for an estrogenic MOA, since there are no other chemically linked biological phenomena known to consistently activate the vitellogenin gene or elevate vitellogenin protein in male fish. Further, since the vitellogenin gene is quiescent in male fish, which implies a zero baseline of vitellogenin, the response is unambiguous with regard to exposure. Additionally, this exposure mediated induction of *vtg* is sensitive to low levels of exogenous estrogen. Because vitellogenin protein has been frequently evaluated in fish studies, accurate methods of measurement (including several commercial kits) are available for many fish species, including the small fish models for which much of the EE2 chronic toxicity data exist. Given these attributes, male VTG has and should continue to be a very useful endpoint for monitoring the occurrence of estrogenic chemicals (including EE2) in the environment. A major drawback to using male-specific circulating protein to assess exposure and risk of EE2 (or other estrogens), including the derivation of ALC, is the lack of an established functional linkage between expression of the protein and adverse endpoints related to early development or reproduction (Wheeler et al. 2005). This is in large part due to the fact that VTG plays no physiological role in male reproductive processes. As such, any associations that might exist between VTG induction in males and reproductive success is likely more correlative than causal.

Despite the fact that the appearance of VTG in males appears not to be a robust predictor of adverse effects on reproduction, the response could nonetheless play an important role in reducing the uncertainty of ALC for EE2, or the development of ALC for other chemicals which

might be estrogenic. From Table 3.2 above, it is apparent that data from life-cycle tests with fish would be appropriate (and critical) to setting the final ALC for EE2 and, by extension, other estrogens. Hence, knowledge that a less well-studied chemical than EE2 induces VTG in males could be used to help identify those instances when one (or more) life-cycle fish assay(s) would be recommended for generating robust data for ALC derivation. Another possible use of protein data, that could have more direct applicability to developing an ALC for EE2, involves use of the endpoint as a basis for evaluating relative species sensitivity. Specifically, reproductive data suitable for an EE2 ALC are largely from three species: fathead minnow, medaka and zebrafish (Table 3.2); however, there are studies with numerous species that have evaluated the ability of EE2 to induce vitellogenin mRNA and protein in males. Provided that a common dose metric could be established across these studies, a dataset could be developed to provide an indication of the relative sensitivity distribution of fish species to EE2, in addition to other estrogens. This would enable a direct comparison of values along the continuum of estrogen sensitivity for those fish species wherein chronic data exist (based on VTG induction) and, as such, could provide a quantitative indication of uncertainty for a proposed EE2 criterion.

There are two mechanism-specific endpoints that have been measured in a number of EE2 studies that might, with additional research and analysis, have a direct bearing on criteria derivation: alterations in sex ratio (i.e., generation of genotypic males with a female phenotype) and the occurrence of intersex/testis-ova (Tables A.5, A.6). As opposed to VTG induction in males, the functional linkage between skewed sex ratios or abnormal gonad development and reproductive success in fish, at both the individual and population levels, is readily apparent. Specific endpoints, however, can be difficult to measure. For example, detailed histological analyses are needed to identify and, especially, quantify testis-ova. To detect an alteration in sex ratio, a genotypic marker of gender (available in medaka but not fathead minnow or zebrafish) or a relatively large representative sample is required to reliably detect chemically-induced changes within a proportion of males and females in a population. Probably more difficult than measurement of the endpoints is definition of the quantitative linkage between changes in sex ratio or occurrence of testis-ova and effects on reproductive success for individuals and populations. For example, unless one assumes that any deviation in sex ratio from the norm (e.g., 1:1) is adverse, it is necessary to know (in the case of estrogenic effects) the magnitude of shift in respective gender numbers that is likely to result in cases where fewer young are produced. Similarly, it is probable that some degree of testis-ova would not be considered adverse in terms of reducing reproductive success, especially considering that the condition can exist at some degree, even in control animals (Grim et al. 2007). It is certain that at some level of manifestation, the condition will impair gonad function sufficiently such that acceptable levels of normal sperm cannot be produced. The frequency of this phenomenon, however, is currently unknown for any fish species. Definition of this relationship would support use of testis-ova occurrence in fish not only for prospective assessments (like criterion derivation), but in environmental monitoring studies focused on chemicals with an estrogenic MOA.

At present, uncertainties regarding measurement and interpretation hamper use of data from any of the mechanism-specific endpoints mentioned above as a basis for derivation of an ALC for EE2. Eith appropriate research, however, induction of vitellogenin in males, changes in sex

ratios and occurrence of testis-ova, all have the potential to contribute insights to different facets of quantitative risk assessment for estrogenic chemicals, including derivation of ALC. There is one noteworthy additional observation relative to use of non-traditional endpoints for an EE2 ALC. Several fish life-cycle studies using EE2 have been conducted in which typical measures of reproductive success (e.g., fecundity, fertility) have been made in conjunction with induction of VTG, sex ratio and/or testis-ova data (e.g., Länge et al 2001; Wenzel et al. 2001; Nash et al. 2004; Parrott and Blunt 2005). Although experimental design variables make some of the endpoint sensitivity comparisons challenging, it does not appear that there are substantial differences in EE2 test concentrations that produce adverse effects on egg production/fertility, versus those that alter the mechanism-specific endpoints. Hence from a pragmatic perspective, at least for the near-term, it seems reasonable to base an EE2 ALC on traditional measures of long-term reproductive success in fish.

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APPENDIX A

Table A.1. Effects of EE2 on Aquatic Animals (Short-term survival).

Species	Life stage	Method	Duration	EC50 or LC50 (ng/L)	Reference	Remarks
Traditional Acute (1-7 day timeframe)						
*Sea urchin, *Strongylocentrotus purpuratus*	embryo	S,U	96 h	30,000	Roepke 2005	EC50 - abnormal development
*Sea urchin, *Lytechinus anamesus*	embryo	S,U	96 h	30,000	Roepke 2005	EC50 - abnormal development
*Copepod, *Acartia tonsa*	egg	R,U	5 d	88,000	Andersen et al. 2001	EC50-Inhibition of naupliar development
Medaka, *Oryzias latipes*	adult	-	96 h	>1,000,000	Thompson 2000	
*Copepod, *Acartia tonsa*	10-12 d adult	S,U	48 h	1,100,000	Andersen et al. 2001	
*Opossum shrimp, *Neomysis integer*	Juv, 2-4 mm	R, U	96 h	1,200,000	Verslycke et al. 2004	
Zebrafish, *Danio rerio*	adult	-	96 h	1,700,000	Wenzel et al. 2001	
Cladoceran, *Ceriodaphnia reticulata*		S,U	24 h	1,800,000	Jaser et al. 2003	EC50 mobility
Cnidarian, *Hydra vulgaris*	Adult male	R, U	96 h	3,800,000	Pascoe et al. 2002	
Cladoceran, *Sida crystallina*		S,U	24 h	>4,100,000	Jaser et al. 2003	EC50 mobility
Cladoceran, *Daphnia magna*	<24 h	S,U	48 h	>5,000,000	Goto and Hiromi 2003	
Midge, *Chironomus riparius*	4th instar	S,M	24 h	9,100,000	Lee and Choi 2006	

*Indicates saltwater species.

Table A.2. Effects of EE2 on Aquatic Animals (Long-term survival).

Species	Life Stage	Method	Duration	NOEC-survival (ng/L)	LOEC - survival (ng/L)	Reference	Remarks
Significant Effect Observed							
Zebrafish, *Danio rerio*	1 d old	R,U	38 d	10	100	Orn et al 2006	100% mortality at LOEC
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	10	100	Hill and Janz 2003	90% mortality at LOEC (45% control mortality); excess solvent
Medaka, *Oryzias latipes*	1 d	R,M	LC: 85 to 110 dph	29	290	Metcalfe et al. 2001	83% mortality at LOEC
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC:59-7 dph F1	120	330	Zillioux et al. 2001	50% mortality at LOEC (42 days)
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	260	490	Seki et al. 2002	42% mortality at LOEC (4 of 5 dead males)
Rainbow trout, *Oncorhynchus mykiss*	1+ year	F,M	62 d pre-spawning	130	750	Schultz et al. 2003	100% mortality at LOEC (57 days)
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	500	2,000	Thompson 2000; Tilton et al. 2005	Significant mortality at LOEC
Zebrafish, *Danio rerio*	fert. eggs	R,U	5 wk	-	5,000	Ortiz-Zarragoitia et al. 2006	50% mortalty of exposed animals
*Copepod, *Tisbe battagliai*	<24 h	R,U	10 d	-	>100,000	Hutchinson et al. 1999	Value is an LC50
Wood frog, *Rana sylvatica*	Gosner 26	R,U	14 d	-	560,000	Hogan et al. 2006	Value is an LC50
Amphipod, *Gammarus pulex*	3-5 mm	R,M	10 d	-	840,000	Watts et al. 2001	Value is an LC50
Leopard frog, *Rana pipiens*	Gosner 26	R,U	14 d	-	890,000	Hogan et al. 2006	Value is an LC50
Leopard frog, *Rana pipiens*	Gosner 36	R,U	14 d	-	1,200,000	Hogan et al. 2006	Value is an LC50
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)							
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	6	-	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	10	-	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	juvenile	F,M	21 d	20	-	Panter et al. 2002	Conc. only 40% of nominal

Species	Life Stage	Method	Duration	NOEC-survival (ng/L)	LOEC - survival (ng/L)	Reference	Remarks
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	25	-	Zha et al. 2007	10% mortality
Zebrafish, *Danio rerio*	fert. eggs	F,U	3 mo	25	-	Van den Belt et al. 2003	40% mortality after 5 mo. recovery
Fathead minnow, *Pimephales promelas*	mature male	F,U	35 d	50	-	Schmid et al. 2002	
Snail, *Potamopyrgus antipodarum*	adult	R,U	9 wk	100	-	Jobling et al. 2004	
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	100	-	Scholz and Gutzeit 2000	8% mortality
Zebrafish, *Danio rerio*	4 wk	R,U	33 d	100	-	Versonnen and Janssen 2004	6.6% mortality; excessive carrier solvent
Guppy, *Poecilia reticulate* (male)	< 7 d	F,M	108 d	110	-	Nielsen and Baatrup 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	120	-	Palace et al. 2001	
Rainbow trout male, *Oncorhynchus mykiss*		F,M	3 wk	130	-	Hook et al. 2007	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	2,960	-	Urbatzka et al. 2007	
Wood frog, *Rana sylvatica*	Gosner stage 25	R,M	76 d	4,100	-	Mackenzie et al. 2003	
Amphipod *Gammarus pulex*	mixed age	F,M	100 d	7,600	-	Watts et al. 2002	
Amphipod adult, *Hyalella azteca*	pre-copulatory	R,M	10 wk - 2 x gen	10,000	-	Vandenbergh et al. 2003	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	100,000	-	Hutchinson et al. 1999	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	100,000	-	Pounds et al. 2002	
Cladoceran, *Sida crystallina*		R,U	34 d	500,000	-	Jaser et al. 2003	
Cladoceran, *Daphnia magna*		R,U	25 d	500,000	-	Goto and Hiromi 2003	

*Indicates saltwater species.

Table A.3. Effects of EE2 on Aquatic Animals (Growth).

Species Significant Effects Observed	Life Stage	Method	Duration	NOEC-growth (ng/L)	LOEC-growth (ng/L)	Reference	Remarks
Zebrafish , *Danio rerio*	fert egg	F,M	LC – F1	0.10	0.3	Wenzel et al. 2001	7% reduction at LOEC (75 dph)
Zebrafish , *Danio rerio*	fert egg	F,M	PLC	0.30	1.1	Wenzel et al. 2001	2% reduction at LOEC (78 dph)
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	0.60	1.5	Orn et al. 2003	**Increased** juvenile wet weight
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	0.76	2.8	Länge et al. 2001	
Zebrafish , *Danio rerio*	fert egg	F,U	3 mo	1	10	Van den Belt et al. 2003	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	1	10	Lin and Janz 2006	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	-	12	Bogers et al. 2006b	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 days	5	25	Zha et al. 2007	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	10	32	Parrot and Wood 2002	
Zebrafish , *Danio rerio*	4 wk	R,U	33 d	10	100	Versonnen and Janssen 2004	Excessive carrier solvent
Guppy, *Poecilia reticulata*	<7d	F,M	108 d	44	112	Nielsen and Baatrup 2006	**Increased** adult wet weight.
Medaka, *Oryzias latipes*	1 day old	R,M	LC	29	290	Metcalfe et al. 2001	
Midge, *Chironomus riparius*	4th instar	S,U	48 hr	50	500	Lee et al. 2006	**Increased** larval dry weight
Midge, *Chironomus riparius*	1st instar	R,M	egg - pupa	100,000	1,000,000	Watts et al. 2003	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)							
Zebrafish, *Danio rerio*	fert egg	F,M	2xGen	4.5	-	Nash et al. 2004	
*Three-spined stickleback, *Gasterosteus aculeatus*	fry	R,U	14 days	7.3	-	Hahlbeck et al. 2004b	

Species	Life Stage	Method	Duration	NOEC-growth (ng/L)	LOEC-growth (ng/L)	Reference	Remarks
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	10	-	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	juv	F,M	21 days	20	-	Panter et al. 2002	
Prosobranch mollusc, *Potamopyrgus antipodarum*	adult	R,U	9 wk	100	-	Jobling et al. 2004	
*Sheepshead minnow , *Cyprinodon variegatus*	juv	F,M	PLC	330	-	Zillioux et al. 2001	
Wood frog , *Rana sylvatica*	Gosner stage 25	R,M	76 d	4,100	-	Mackenzie et al. 2003	

*Indicates saltwater species.

Table A.4. Chronic Reproductive Effects of EE2 on Aquatic Animals (Fecundity, Fertility, and Population Growth).

Species	Life Stage	Method	Duration	Endpoint	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Percent fertilized of eggs laid	-	0.32	Parrott and Blunt 2005	
Zebrafish, *Danio rerio*	fert egg	F,M	LC	Number of fertilized eggs per female	0.30	1.1	Wenzel et al. 2001	
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	Mean no. eggs laid per breeding day	0.76	2.8	Länge et al. 2001	
Zebrafish, *Danio rerio*	fert egg	F,M	118 d	No. eggs spawned and prop fertilized	-	3	Fenske et al. 2005	
Fathead minnows, *Pimephales promelas*	-	Field	3 yrs	Population crash	-	3.2 – 8.9	Kidd et al. 2007	
Green frog, *Rana clamitans*	fert egg	Field	2 yr	Hatching success	-	3.2 – 8.9	Park and Kidd 2005	
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Proportion of non-viable eggs	0.50	4.5	Nash et al. 2004	Complete Rep. failure at LOEC
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 months	Fertile eggs and hatching success	-	6	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	6-11 mo.	F,M	3 wk	Fert rate and no. eggs spawned	0.75	7.5	Pawlowski et al. 2004	**Increased** No. eggs spawned up to 0.75 ng/L
Zebrafish, *Danio rerio*	2 dph	R,U	60 d	% viable eggs, % hatch, % swim-up	1	10	Hill and Janz 2003	Excessive carrier solvent
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	Female egg production	1	10	Scholz and Gutzeit 2000	No effect on male fert at 10 ng/L
Zebrafish, *Danio rerio*	fert egg	F,U	3 mo	No. spawning females & egg prod	1	10	Van den Belt et al. 2003	
Zebrafish, *Danio rerio*	8 mo	R,U	14 d	Absence of intact eggs in ovaries	1	10	Versonnen and Janssen 2004	
Rainbow trout, *Oncorhynchus mykiss*	1+ year	F,M	PLC	Fertilization success	-	16	Schultz et al. 2003	EC50; same response@131 ng/L
Snail, *Potamopyrgus antipodarum*	adult	R,U	9 wk	Embryo production	25	100	Jobling et al. 2004	EE2 at 25 ng/L stimulatory
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC	Hatching success	18	120	Zillioux et al. 2001	
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	Fecundity	260	490	Seki et al. 2002	

Species	Life Stage	Method	Duration	Endpoint	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	Spawning frequency, % fertilized and % hatch.	5	500	Thompson 2000; Tilton et al. 2005	
Cladoceran, *Daphnia magna*	-	R,U	25 d	Embryo production	20,000	100,000	Goto and Hiromi 2003	
Rotifer, *Brachionus calyciflorus*		S,U	72 hr	Ratio of ovigerous/non-ovigerous females	202,000	510,000	Radix et al. 2002	
Rotifer, *Brachionus calyciflorus*		S,U	72 hr	Intrinsic rate population increase r	510,000	1,300,000	Radix et al. 2002	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Fathead minnow, *Pimephales promelas*	>6 mo.	F,M	3 wk	No. spawnings and eggs per spawn	1.5	-	Brian et al. 2007	
Mink frog, *Rana septentrionalis*	fert egg	Field	2 yr	Hatching success	3.2 – 8.9	-	Park and Kidd 2005	
Zebrafish, *Danio rerio* (females)	5-6 mo	R,U	15 d	Sterility in females	5,000	-	Ortiz-Zarragoitia et al. 2006	
Amphipod, *Gammarus pulex*	mixed ages	F, M	100 d	Population growth (total pop. size)	7,600	-	Watts et al. 2002	**Increase** in population size
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 days	Fecundity	100,000	-	Hutchinson et al. 1999	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 days	Reproduction	100,000	-	Pounds et al. 2002	

*Indicates saltwater species.

Table A.5. Chronic Reproductive Effects of EE2 on Aquatic Animals (Sex Reversal).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
Zebrafish, *Danio rerio*	fert egg	F,U	3 mo	Delayed sexual differentiation	-	0.10	Van den Belt et al. 2003	No males at LOEC
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	Male:female sex ratios	-	0.60	Orn et al. 2003	Complete sex reversal at 1.5 ng/L
Fathead minnow, *Pimephales promelas*	40-60 h old	F,M	LC	Male:femal sex ratio	0.32	0.96	Parrott and Blunt 2005	Complete ex. femin. at 3.5 ng/L
Fathead minnow, *Pimephales promelas*	fert. eggs	F,U	60 d	Male:female sex ratio	0.32	1.0	Parrot and Wood 2002	Complete ex. femin. at 3.2 ng/L
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Male:female sex ratio	-	1.0	Lin and Janz 2006	
Fathead minnow, *Pimephales promelas*	<24 hr	F,M	LC	Sex reversal - all female	0.76	2.8	Länge et al. 2001	Sex ratio at 0.76 ng/L 54:46
Zebrafish, *Danio rerio*	fert egg	F,M	42 d	Male feminization	-	3.0	Fenske et al. 2005	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Sex reversal - all female	-	10	Parrot et al. 2003	
Zebrafish, *Danio rerio*	1 dph	R,U	60 d	Complete feminization	-	10	Orn et al 2006	
Zebrafish, *Danio rerio*	1 dph	R,U	60 d	Complete feminization	-	10	Yamani 2004	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	75% female gonads; 15% un-developed	-	12	Bogers et al. 2006b	
Zebrafish, *Danio rerio*	fert egg	R,M	60 d	Complete feminization	-	15	Andersen et al. 2003b	
Medaka, *Oryzias latipes*	1 d	R,M	LC	Male:female sex ratio	2.9	29	Metcalfe et al. 2001	
*Three-spined stickleback, *Gasterosteus aculeatus*	Larvae	R,U	42 d	Sex reversal and intersex	-	50	Hahlbeck et al. 2004a	
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	Sex reversal with ovary	10	100	Scholz and Gutzeit 2000	
Medaka, *Oryzias latipes*	1 d	R,U	60 d	88% female, 2% male, 10% intersex	10	100	Yamani 2004 and Orn et al 2006	
Amphipod, *Gammarus pulex*	mixed ages	F, M	100 d	Male:femal ratio	-	104	Watts et al. 2002	No dose-response >104 ng/L

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Guppy, *Poecilia reticulate*	< 7 d	F,M	108 d	Male:female sex ratio	44	110	Nielsen and Baatrup 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Green frog, *Rana clamitans*	fert egg	Field	2 yr	Male:female sex ratio	3.2 – 8.9	-	Park and Kidd 2005	
Mink frog, *Rana septentrionalis*	fert egg	Field	2 yr	Male:female sex ratio	3.2 – 8.9	-	Park and Kidd 2005	
Amphipod, *Hyalella azteca*	adult	R,M	10 wk; 2 gen	Male:femal sex ratio	10,000	-	Vandenbergh et al. 2003	
*Copepod, *Tisbe battagliai*	<24 hr	R,U	21 d	Male:female sex ratio	100,000	-	Hutchinson et al. 1999	
Cladoceran, *Daphnia magna*	-	R,U	25 d	Male:female ratio	500,000	-	Goto and Hiromi 2003	

*Indicates saltwater species.

Table A.6. Chronic Reproductive Effects of EE2 on Aquatic Animals (Intersex).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed								
Pearl dace, *Margariscus margarita*	mature	Field	3 yr	Presence of testis-ova	-	3.2 – 8.9	Palace et al. 2006	Edema in ovaries
Fathead minnows, *Pimephales promelas*		Field	3 yr	Presence of testis-ova	-	3.2 – 8.9	Kidd et al. 2007	Testicular malformations
Mink frog, *Rana septentrionalis*	fert eggs	Field	2 yr	Intersex gonads (5 – 12 %)	-	3.2 – 8.9	Park and Kidd 2005	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Testis-ova in males	1.0	5.0	Zha et al. 2007	No sperm detectable
Fathead minnow, *Pimephales promelas*	egg	F,U	5 d	Ovarian cavities in males (8%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	5-10 dph	F,U	5 d	Ovarian cavities in males (38%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	10-15 dph	F,U	5 d	Ovarian cavities in males (64%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	15-20 dph	F,U	5 d	Ovarian cavities in males (43%)	-	10	Van Aerle et al. 2002	
Fathead minnow, *Pimephales promelas*	egg	F,U	20 d	Ovarian cavities in males (22%)	-	10	Van Aerle et al. 2002	
Medaka, *Oryzias latipes*	1 d	R,M	LC	Sex inversion and testis-ova	2.9	29	Metcalfe et al. 2001	4 of 4 males with TO
*Three-spined stickleback, *Gasterosteus aculeatus*	Larvae	R,U	42 d	Intersexed gonads	-	50	Hahlbeck et al. 2004a	
Medaka, *Oryzias latipes*	6 mo	F,M	21 d	Testis-ova in males (33%)	33	64	Seki et al. 2002	No histological abnormalities in females
Medaka, *Oryzias latipes*	1 d	R,U	2 mo	All males developed an ovary	10	100	Scholz and Gutzeit 2000	No effect on male fertility
Medaka, *Oryzias latipes*	1 d	R,U	60 d	Intersexed gonads (10%)	10	100	Yamani 2004 and Orn et al 2006	
Guppy, *Poecilia reticulata*	< 7 d	F,M	108 d	Feminization of male reproductive ducts	44	110	Nielsen and Baatrup 2006	
Amphipod, *Hyalella azteca*	fert eggs	R,M	2 x gen	Oocyte-like structures in males	23	320	Vandenbergh et al. 2003	
Leopard frog, *Rana pipiens*	Gosner 25	R,M	162 d	Intersex and altered testicular develepment	414	4,140	Mackenzie et al. 2003	

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Wood frog, *Rana sylvatica*	Gosner 25-28	R,M	76 d	Intersex and altered testicular development	-	4,140	Mackenzie et al. 2003	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Green frog, *Rana clamitans*	fert eggs	Field	2 yr	Intersexed gonads	3.2 - 8.9	-	Park and Kidd 2005	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Testis-ova	10	-	Lin and Janz 2006	
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Testis-ova	10	-	Hill and Janz 2003	Excessive carrier solvent; high control mortality
Zebrafish, *Danio rerio*	adult	F,U	21 d	Feminization of testes	25	-	Islinger et al. 2003	

*Indicates saltwater species.

Table A.7. Chronic Reproductive Effects of EE2 on Aquatic Animals (Sexual Behavior).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Remarks
Significant Effects Observed								
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	Male nesting behavior	-	6	Robinson et al. 2003	High mortality in solvent controls in first month of exposure
Fathead minnow, *Pimephales promelas*	mature	F,M	27 d	Impaired ability to compete and acquire territory	2.0	8.9	Majewski et al. 2002	
*Three-spined stickleback, *Gasterosterus aculeatus*	mature	R,U	12 d	Time spent near nest and glueing frequency, but effect short-lived	-	10	Brian et al. 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Natural spawning behavior of adult male fish	4.5	-	Nash et al. 2004	Sexually compromised males still actively participated in the spawning act, i.e., chasing females and competing with healthy males
Amphipod, *Gammarus pulex*	3-5 mm	R,M	10 d	Pre-copulatory guarding behavior	3,700,000	-	Watts et al. 2001	Reproductive behavior was only disrupted at high concentrations where it would be unrealistic to attribute effects to and endocrine-mediated process.

Table A.8. Data on Effects of EE2 on Aquatic Animals (Secondary Sexual Characterstics).

Species	Life Stage	Method	Duration	Effect	Event Association	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed									
Fathead minnow, *Pimephales promelas*	6-11 mo.	F,M	3 wk	Number of male nuptial tubercles	Activational	-	0.80	Pawlowski et al. 2004	
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Ovipositor size, nuptial tubercles, banding strength	Organizational	0.32	0.96	Parrott and Blunt 2005	
Fathead minnow, *Pimephales promelas*	156 dph	F,M	LC	Nuptial tubercles, banding strength, dorsal fin dot, dorsal fat pad	Activational	0.32	0.96	Parrott and Blunt 2005	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	Male sex index: nuptial tubercles, dorsal fat pad, dorsal fin dot, banding strength	Organizational	0.32	1.0	Parrot and Wood 2002	Complete femin. at 3.2 ng/L
Fathead minnow), *Pimephales promelas*	<24 hr	F,M	LC	Secondary sex characteristics – not specified	Organizational	0.76	2.8	Länge et al. 2001	50% sex ratio at 0.76 ng/L
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	60 dph	Development and length of ovipositors	Organizational	1.0	3.2	Parrot and Wood 2002	Complete femin. at 3.5 ng/L
Zebrafish, *Danio rerio*	fert egg	F,M	2 x gen	Coloration and bright anal fin markings	Organizational	-	4.5	Nash et al. 2004	
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	Delayed and inhibited nuptial coloration in males	Activational	-	6.0	Robinson et al. 2003	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Ovipositor size, nuptial tubercles, banding strength	Organizational	-	10	Parrot et al. 2003	
Fathead minnow, *Pimephales promelas*	maturing	F,M	21 d	Number and prominence of nuptial tubercles and dorsal fat pad	Activational	-	11	Filby et al. 2007	
Fathead minnow, *Pimephales promelas*	embryo	F,M	114 d	Number and prominence of nuptial tubercles	Organizational	-	12	Bogers et al. 2006b	
Amphipod, *Hyalella azteca*	fert eggs	R,M	2 x gen	Male second gnathopods	Organizational	-	23	Vandenbergh et al. 2003	No effect >1,000 ng/L
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)									
Fathead minnow, *Pimephales promelas*	>6 mo.	F,M	3 wk	Relative fat pad weight, number of nuptial tubercles, nuptial tubercle prominence	Activational	1.5	-	Brian et al. 2007	
Medaka, *Oryzias latipes*	4 mo	R,U	14 d	Anal and dorsal fin shape	Organizational	500	-	Thompson 2000; Tilton et al. 2005	

*Indicates saltwater species.

Table A.9. Chronic Reproductive Effects of EE2 on Aquatic Animals (Vitellogenin).

Species	Life Stage	Method	Duration	Effect	NOEC (ng/L)	LOEC (ng/L)	Reference	Comments
Significant Effects Observed								
Zebrafish, *Danio rerio*	adult males	F,M	40 d	Increased whole-blood VTG level	-	0.50	Nash et al. 2004	
Fathead minnow, *Pimephales promelas*	6-11 mo	F,M	3 wk	Increased plasma VTG levels	-	0.80	Pawlowski et al. 2004	
Rainbow trout, *Oncorhynchus mykiss*	immature female	F,M	14 d	Increased liver *vtg* mRNA expression and plasma VTG	0.21	1.0	Thomas-Jones et al. 2003	
Zebrafish, *Danio rerio*	20 dph	R,M	40 d	Increased whole body VTG levels	-	1.5	Orn et al. 2003	
Zebrafish ,*Danio rerio*	adult male	R,U	21 d	Increased plasma VTG level	-	1.6	Fenske et al. 2001	
Rainbow trout, *Oncorhynchus mykiss*	Adult male	F,M	3 wk	Increased plasma VTG level	-	1.8	Jobling et al. 1996	
Zebrafish, *Danio rerio*	fert egg	F,M	42 d	Increased vitellogenin level	-	3.0	Fenske et al. 2005	
Fathead minnow, *Pimephales promelas*	-	Field	5 mo	Increased plasma VTG levels	-	3.2 – 8.9	Palace et al. 2002 (also see Kidd et al. 2007)	
Pearl dace, *Margariscus margarita*	-	Field	3 yrs	Increased whole body VTG level	-	3.2 – 8.9	Palace et al. 2006	
Zebrafish, *Danio rerio*	adult male	F,M	8 d	Increased whole-body VTG level	2.2	3.6	Rose et al. 2002	EC10 = 0.92 ng/L
Zebrafish, *Danio rerio*	adult females	F,M	40 d	Increased whole-blood VTG level	0.50	4.5	Nash et al. 2004	
Fathead minnow, *Pimephales promelas*	juv	F,M	21 d	Increased whole-body VTG level	2.0	5.0	Panter et al. 2002	
Fathead minnow, *Pimephales promelas*	8 mo	R,M	48 hrs	Increased liver *vtg* levels	2.5	5.0	Biales et al. 2007	
Ide, *Leucisus idus*	juv	F,M	7 d	Increased plasma VTG levels	-	6.0	Allner et al. 1999	
Zebrafish, *Danio rerio*	fert egg	R,M	4 d	Increased whole body VTG level	2.6	7.8	Bogers et al. 2006a	
Zebrafish, *Danio rerio*	adult females	R,M	21 d	Increased plasma VTG level	4.1	8.5	Van den Belt et al. 2004	
Zebrafish, *Danio rerio*	adult males	F,M	24 d	Increased plasma VTG level	-	9.0	Van den Belt et al. 2002	

Rainbow trout, *Oncorhynchus mykiss*	11 mo	F,M	2 wk	Increased plasm VTG level	0.87	10	Samuelsson et al. 2006	
*Eelpout, *Zoarces viviparus*	adult female	F,M	3 wk	Increased plasma VTG level	5.0	10	Korsgaard et al. 2002	
Fathead minnow), *Pimephales promelas*	<24 hr	F,M	LC	Increased whole body VTG level	2.8	12	Länge et al. 2001	
Zebrafish, *Danio rerio*	adults	F,M	168 hrs	Increased plasma VTG level	-	14	Hoffmann et al. 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	Increased plasma VTG levels	-	14	Palace et al. 2001	
Lake trout, *Salvelinus namaycush*	immature	F,M	21 d	Increased plasma VTG level	-	15	Werner et al. 2003	Excessive carrier solvent
*Baltic flounder, *Platichthys flesus*	adult	F,M	21 d	Increased plasma VTG level in male and female fish	-	15	Allen et al. 1999b	
Medaka, *Oryzias latipes*	6 mo.	F,M	21 d	Increased liver Vtg levels	33	64	Seki et al. 2002	
Rainbow trout, *Oncorhynchus mykiss*	juvenile	R,M	14 d	Increased plasma VTG level	10	100	Verslycke et al. 2002	
*Sheepshead minnow, *Cyprinodon variegatus*	male	F,M	16 d	Increased liver *vtg* mRNA expression	24	110	Folmar et al. 2000	
Rainbow trout, *Oncorhynchus mykiss*	mature male	F,M	61 d	Increased plasma VTG levels	-	140	Skillman et al. 2006	
No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)								
Zebrafish, *Danio rerio*	adult males	F,M	310 dpf F1	No effect on whole-blood VTG level	4.5	-	Nash et al. 2004	
Zebrafish, *Danio rerio*	adult females	F,M	310 dpf F1	No effect on whole-blood VTG level	4.5	-	Nash et al. 2004	

*Indicates saltwater species.

Table A.10. Chronic Effects of EE2 on Aquatic Animals (Other Relevant Endpoints).

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
Significant Effects Observed							
Zebrafish , *Danio rerio*	17-20 dpf	R,U	3 d	Enhanced effect on CYP19A2 gene expression	0.30	Kazeto et al. 2004	Excessive carrier solvent
Zebrafish, *Danio rerio*	2 dph	R,U	58 d	Suppression of gametogenesis for males (no testes discernable) and females	1.0	Weber et al 2003	Excessive carrier solvent
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased GSI and renal somatic index (RSI) in males	1.0	Zha et al. 2007	
Fathead minnow, *Pimephales promelas*	40-60 h	F,M	LC	Reduced GSI in females	3.5	Parrott and Blunt 2005	
Atlantic salmon, *Salmo salar*	immature	S,U	7 d	Increased AchE and GST activities and lactate content after 3 days, but no effect at 7 days	5.0	Greco et al. 2007	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Reduced GSI in females	5.0	Zha et al. 2007	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased RSI in females	5.0	Zha et al. 2007	
Medaka, *Oryzias latipes*	4 mo	R,U	14 d	Increased male and female plasma E2 levels	5.0	Thomposn 2000; Tilton et al. 2005	
Fathead minnow, *Pimephales promelas*	6-11 mo	F,M	3 wk	Reduction on male GSI	7.5	Pawlowski et al. 2004	
Zebrafish, *Danio rerio*	Adult female	R,M	21 d	Reduced female ovarian somatic index	8.5	Van den Belt et al. 2004	
Rainbow trout, *Oncorhynchus mykiss*	11 mo	F,M	2 wk	Higher hepatosomatic index (HSI)	10	Samuelsson et al. 2006	
Fathead minnow, *Pimephales promelas*	fert eggs	F,U	125 d	Increased liver somatic index	10	Parrot et al. 2003	
Medaka, *Oryzias latipes*	1 d	R,U	4 mo	In both males and females, significantly increased number of necrotic hepatocytes and kidney tubule cells	10	Weber et al. 2004	
*Baltic flounder, *Platichthys flesus*	adult	F,M	21 d	Increased HSI in males	15	Allen et al. 1999	
Zebrafish, *Danio rerio*	adult male	S,M	21 d	Increased levels of cyp19a2 mRNA (aromatase)	21	Kallivretaki et al. 2006	
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Increased HSI in males	25	Zha et al. 2007	

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
Chinese rare minnow, *Gobiocypris rarus*	7 mo	F,U	28 d	Ovary degeneration in females	25	Zha et al. 2007	
Zebrafish, Danio rerio	adult male	F,M	7 d	Decreased testrosterone and 11-ketotestosterone levels	26	Andersen et al. 2006	
Atlantic salmon, *Salmo salar*	immature	S,U	72 hr	Induced expression of brain P450 Aromatase	50	Lyssimachou et al. 2006	
Sturgeon, *Acipenser fulvescens*	1 yr	F,M	25 d	Increased plasma Vit E, A1 and A2; Decreased Vit E and A in kidney	60	Palace et al. 2001	
*Sheepshead minnow, *Cyprinodon variegatus*	juv	F,M	PLC	Increased pathological condition of kidneys	120	Zillioux et al. 2001	Fish survived to reproduction
Rainbow trout, *Oncorhynchus mykiss*	mature male	F,M	3 wk	Changed gene expression profile	130	Hook et al. 2007	
Zebrafish , Danio rerio	18-21 d	R,U	72 hr	Stimulated expression of Cytochrome P450 aromatase (Aro-B)	300	Le Page et al. 2006	
Medaka, *Oryzias latipes*	mature	R,U	14 d	Induced ER protein and aromatase activity	500	Contractor et al. 2004	
Medaka, *Oryzias latipes*	adult	R,U	14 d	Increased hepatic estrogen receptor (ER)	500	Thompson 2000	
Medaka, *Oryzias latipes*	4 mo.	R,U	14 d	Decreased female and male GSI	500	Thompson 2000; Tilton et al. 2005	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	Reduced Leutinizing hormone B mRNA expression	3,000	Urbatzka et al. 2006	
African clawed frog, Xenopus laevis	adult	R,U	4 wk	Reduced testosterone levels in both sexes	3,000	Urbatzka et al. 2007	
African clawed frog, Xenopus laevis	adult	R,U	4 wk	Reduced E2 level in females	3,000	Urbatzka et al. 2007	
Midge, *Chironomus riparius*	4th instar	S,U	24 h	Increased expression of heat shock proteins	8,000	Lee et al. 2006	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin L	10,000	Lee et al. 2002b	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin H levels	20,000	Lee et al. 2002b	
Medaka, *Oryzias latipes*	mature male	R,U	6 d	Increased mRNA expression of liver choriogenin H levels	20,000	Lee et al. 2002a	
Medaka, *Oryzias latipes*	juv	R,U	6 d	Increased mRNA expression of whole body Choriogenic H	50,000	Lee et al. 2002a	

No Signficiant Effects Observed (NOEC Equals Highest Test Concentration)

Species	Life Stage	Method	Duration	Effect	Concentration (ng/L)	Reference	Remarks
*Sand goby, *Pomatoschistus minutus*	juv	F,U	7 mo	No effect on GSI in males or females	6.0	Robinson et al. 2003	
Rainbow trout, *Oncorhynchus mykiss*	juvenile	R,M	14 d	No effect on GSI or HSI	100	Verslycke et al. 2002	
African clawed frog, *Xenopus laevis*	adult	R,U	4 wk	No effect on Gonadotropin Releasing Hormone mRNA expression	3,000	Urbatzka et al. 2006	

*Indicates saltwater species.

Exhibit 3

Analysis and removal of emerging contaminants in wastewater and drinking water

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Abstract

The occurrence of trace organic contaminants in wastewaters, their behaviour during wastewater treatment and drinking water production are the key issues in relation to the reuse of water resources. Elimination of different classes of emerging contaminants, such as surfactant degradates, pharmaceuticals and polar pesticides in wastewater treatment plants (WWTP) was found to be rather low and consequently sewage effluents are one of the main sources of these compounds and their recalcitrant metabolites.

This paper reviews the state-of-the-art in the analysis of several groups of emerging contaminants (acidic pharmaceuticals, antibacterial agents, acidic pesticides and surfactant metabolites) in wastewaters. Their elimination in WWTP applying conventional activated sludge treatment and advanced treatment processes, such as membrane bioreactors (MBR) and advanced oxidation (AOP), as well as the elimination during drinking water production are discussed.

Keywords

Acidic pharmaceuticals; Acidic pesticides; Advanced treatment; Emerging contaminants; Surfactant degradates, Wastewater treatment

1. Introduction

Until the beginning of the 1990' non-polar hazardous compounds, i. e. persistent organic pollutants (POP) and heavy metals, were in the focus of interest and awareness as priority pollutants, and consequently were part of intensive monitoring programs. Today, these compounds are less relevant for the industrialized countries since a drastic reduction of emission has been achieved due to the adoption of appropriate measures and elimination of the dominant pollution sources.

However, the emission of so-called "emerging" or "new" unregulated contaminants has emerged as an environmental problem and there is a widespread consensus that this kind of contamination may require legislative intervention. This group is mainly composed of products used in large quantities in everyday life, such as human and veterinary pharmaceuticals, personal care products, surfactants and surfactants' residues, plasticizers and different industrial additives. The characteristic of these contaminants is that they do not need to be persistent in the environment to cause negative effect since their high transformation/removal rates can be compensated by their continuous introduction into environment. One of the main sources of emerging contaminants are untreated urban wastewaters and wastewater treatment plant (WWTP) effluents. (Fig. 1). Most current WWTP are not designed to treat this type of substances and the high portion of emerging compounds and their metabolites can escape elimination in the WWTP and enter the aquatic environment via sewage effluents.

The partial or complete closing of water cycles is an essential part of sustainable water resources management and the increasing scarcity of pristine waters for drinking water supply and increasing consume of water by industry and agriculture should be countered by the efficient and rational utilisation of resources. One of the options is

increasing reuse of effluents for various purposes, especially in industrial and agro/food production activities. However, due to high cost of end-of-pipe approach (drinking water treatment) the future of indirect potable reuse requires an efficient treatment of wastewaters prior to their discharge. Thus, the occurrence of trace organic contaminants in wastewaters, their behaviour during wastewater treatment and drinking water production are the key issues that require further study.

Many believe that of all emerging contaminants, antibiotics are the biggest concern, since their emission in the environment can result in an increased occurrence of resistant bacteria in the environment [1]. However, other emerging compounds, especially polar one, such as acidic pharmaceuticals, acidic pesticides and acidic metabolites of non-ionic surfactants also deserve particular attention. Due to their physico-chemical properties (high water solubility and often poor degradability) they are able to penetrate through all natural filtration steps and man-made treatments, thus presenting a potential risk for drinking water supply [2,3].

Different classes of emerging contaminants, mainly surfactant degradates, pharmaceuticals and personal care products (PPCP) and polar pesticides were found to have rather low elimination rates and have been detected in WWTP effluents and in the receiving surface waters. However, for most of emerging contaminants, occurrence, risk assessment and ecotoxicological data are not available and it is difficult to predict their fate in the aquatic environment. Partially, the reason for this is a lack of analytical methods for their determination at trace concentrations. Analysis of emerging contaminants is a real analytical challenge, not only because of the diversity of chemical properties of these compounds, but also because of generally low concentrations (usually part per billion or part per trillion levels) and the complexity of matrices.

This paper reviews the state-of-the-art in the analysis of several groups of emerging contaminants (acidic pharmaceuticals, antibacterial agents, acidic pesticides and surfactant metabolites) in wastewaters. Various aspects of current LC-MS-(MS) and GC-MS methodology, including sample preparation, are discussed.

It also gives a survey of their elimination in WWTP by activated sludge treatment (AST) and applying advanced treatment processes, such as membrane bioreactors (MBR) and advanced oxidation (AOP). Additionally, the elimination in treatment processes at drinking water treatment plants is discussed.

2. Analysis of emerging contaminants in wastewaters

One of the major limitations in the analysis of emerging contaminants remains to be the lack of analytical methods for quantification of low concentrations. The prerequisite for proper risk assessment and monitoring of waste, surface and drinking water quality is the availability of a multiresidual analytical method that permits measurement at the low (or even below) ng/L level. However, the fact that these compounds are not on the regulatory lists as environmental pollutants resulted in comparatively little attention received. Consequently, analytical methodology for different groups of emerging contaminants is evolving and the number of methods described in the literature for the determination of emerging contaminants has grown considerably. Still, the analysis of this group of contaminants requires further improvements in terms of sensitivity and selectivity, especially for very complex matrices, such as wastewater.

2.1. Acidic pharmaceuticals

Different analytical methods, mainly based on LC-MS and GC-MS, repectively in combination with either polymer or C_{18}-based solid phase extraction (SPE), are being developed for the analysis of pharmaceutical compounds. However, most methods are tailored for neutral compounds (e.g. antibiotics) and less complex matrices (surface and groundwater), while only a limited number of paper describes procedures applicable to the analysis of polar drugs in wastewater samples. A survey of analytical methods for the quantification of regularly used polar pharmaceuticals in wastewater matrices is given in Table 1.

A typical analytical method includes the use of octadecylsilica, polymeric, or hydrophilic-lipophilic balanced (HBL) supports for off-line SPE of water samples, with either disks or, most frequently, cartridges at low pH (typically pH=2).

Separation technique used includes both GC and LC, while for detection, MS has been the technique most widely employed. Due to low volatility of polar pharmaceuticals GC-MS analysis requires additional derivatization step, which makes the sample preparation laborious and time consuming, and also increases the possibility of contamination and errors. Moreover, some compounds are thermolabile and decompose during GC analysis (e.g. carbamazepine forms iminostilben as degradation product) [4].

Consequently, LC-MS and LC-MS-MS are increasingly used. Reviewing principal analytical methods employed in the analysis of pharmaceuticals in aqueous environmental samples Ternes [4] indicated LC-MS-MS as the technique of choice to assay polar pharmaceuticals and their metabolites, however pointed out the difficulty in the enrichment step, as well as the low resolution and the suppression of signals in the electrospray (ESI) interface due to matrix impurities. Farré et al. [5] have compared LC-(ESI)-MS and GC-

MS (after a derivatization with BF_3-MeOH) for the monitoring of some acidic and very polar analgesics (salicylic acid, ketoprofen, naproxen, diclofenac, ibuprofen and gemfibrozil) in surface waters and wastewater samples. Results showed a good correlation between methods expect for the gemfibrozil which derivatization was not completely achieved in some samples.

In general, the limits of detection (LODs) achieved with the LC-MS-(MS) methods were slightly higher than those obtained with the GC-MS methods (see Table 1), however, LC-MS methodology showed advantages in terms of versatility and less complicated sample preparation (no derivatization needed).

Table 2 summarizes the quantitation and qualifier ions used by the various authors for the determination of polar drugs in wastewaters using the selected ion monitoring (SIM) or the multiple reaction monitoring (MRM) mode. The use of triple-quadropole mass spectrometers in LC analysis has substantially increased the selectivity and sensitivity of the determination, resulting in LODs better than those achieved by use of single-quadropole LC-MS. Acidic drugs were usually detected using an ESI interface under negative ionization conditions and deprotonated molecules where chosen as precursor ions. Typical fragmentation pattern obtained with LC-MS-MS showed a loss of CO_2 (or loss of the acidic moiety), with a limited number of other products. For example, for diclofenac, ibuprofen and ketoprofen the product ions generated by expulsion of CO_2 were the only fragment ions formed.

2.2. Acidic pesticides

Chlorinated phenoxy acid herbicides account for the majority of pesticides used worldwide and their presence in environmental waters is well documented. However, their

behaviour during wastewater treatment was rarely studied. This group of herbicides is characterized by high polarity and thermal lability. For these reasons LC is generally more suitable for their analysis, as the sample pretreatment does not require a time-consuming derivatization step. However, the methods used to determine chlorinated phenoxy acid herbicides are still dominated by GC either with electron capture detector (ECD) or MS detection. The main disadvantage of GC analysis is that requires prior derivatization step, usually using highly toxic and carcinogenic diazomethane or, less frequently used, acid anhydrides, benzyl halides and alkylchloroformates. The injection-port derivatization with an ion-pair reagent has been successfully applied [6], as well as, in-situ derivatization prior to solid-phase microextraction (SPME) [7].

Alternative methods based on LC-MS have been proposed, using an ESI interface, which is well suited to the determination of easily ionized chlorinated phenoxy acids. Using LC-MS-(MS), phenoxy acid herbicides are detected under negative ionization conditions typically yielding $[M-H]^-$ ion and one abundant fragment formed by the loss of acidic moiety [8,9].

Recently, in-tube SPME followed by LC-MS was applied for the determination of six chlorinated phenoxy acid herbicides [10]. However, method was applied to river water achieving LODs ranging from 5 to 30 ng/l, while more complex wastewater matrix was not tested.

2.3. Antiseptics

Several methods have been proposed for the determination of triclosan (5-chloro-2-[2,4-dichlorophenoxy] phenol), which is used as an antiseptic agent in a vast array of

personal care (e.g. toothpaste, acne cream, deodorant, shampoo, toilet soap) and consumer products (children's toys, footwear, kitchen cutting boards).

Methods based on diazomethane derivatization and capillary GC-ECD was applied for their quantification in the wastewater of a slaughterhouse [11], using silica clean up without derivatization and analysis by GC-MS [12] and SPE of acidified wastewater water samples and SFE for lyophilized sludge, respectively followed by derivatization and GC-HRMS were recently developed [13].

Recently, a method based on LC-MS was also proposed, achieving a limit of detection of 0.35 μg/L in spiked urban wastewater [14].

2.4. Alkylphenolic compounds

The trace analysis of alkylphenol ethoxylates (APEOs) and their acidic metabolites by LC-MS or LC-MS-MS using atmospheric pressure ionization (API) has been recently reviewed by Petrovic *et al*. [15,16] and the performances of two ionization methods, APCI and ESI, in terms of selectivity and sensitivity toward oligomeric mixtures of APEOs has been discussed. Generally, ESI interface is more often used for the analysis of alkylphenolic compounds due to the higher sensitivity, especially for alkylphenols and carboxylated compounds.

Alkylphenoxy carboxylates (APEC) were detected, in both, the NI and PI mode. In the NI mode, using ESI, APECs give two types of ions, one corresponding to the deprotonated molecule $[M-H]^-$ (*m/z* 277, 321, 263 and 307 corresponding to nonylphenol carboxylate (NPE_1C), nonylphenol ethoxycarboxylate (NPE_2C), octylphenol carboxylate (OPE_1C), and octylphenol ethoxycarboxylate (OPE_2C), respectively) and the other corresponding to deprotonated alkylphenols [17]. The relative abundance of these two ions

depends on the extraction voltage. In the presence of ammonium acetate and using an APCI under PI conditions NPE_1C gave $[M+NH_4]^+$ ions at *m/z* 296, while NPE_2C, gave $[M+NH_4]^+$ ion at *m/z* 340 [18].

LC-ESI-MS was also applied for the analysis of the dicarboxylated breakdown products (carboxylated alkylphenoxy carboxylates; CAPECs) in wastewaters [19,20]. However, the identification of these compounds using LC-MS, under conditions giving solely molecular ions, is difficult since CA_nPE_mCs have the same molecular mass as APECs having one ethoxy unit less and a shorter alkyl chain ($A_{n-1}PE_{m-1}C$). Moreover, since some compounds partially co-elute, the unequivocal assignment of the individual fragments can be accomplished only using LC-MS-MS. Typical fragmentation pattern obtained with LC-ESI-MS-MS showed the formation of the carboxy-alkylphenoxy fragment, with an additionally lost CO_2, or an acetic acid group, in the case of $CA_5PE_{1-2}C$ leading to the fragments of *m/z* 149 and 133 [19].

MS-MS spectra of APECs [19,21,22] shows an intense signal at *m/z* 219 (for NPECs) and *m/z* 205 (for OPECs) that is produced after the loss of the carboxylated (ethoxy) moiety, while sequential fragmentation of the alkyl chain resulted in ions *m/z* 133 and 147.

To overcome the problem with the low volatility of acidic alkylphenolic compounds different off-line and on-line derivatization protocols, respectively have been developed. Off-line derivatization to corresponding triemethylsilyl ethers, methyl ethers, acetyl esters, pentafluorobenzoyl, or heptafluorobutyl esters, respectively was applied as a common approach in GC-MS analysis. On-line direct GC injection-port derivatization using ion-pair reagents (tetraalkylammonium salts), has been also reported [23]. The most significant ions in GC-(EI)-MS of methylated NPECs were fragments produced by rupture of the benzylic bond in the branched nonyl side-chain [23,24,25]. GC-CI-MS spectra of

the NPECs with isobutane as reagent gas showed characteristic hydride ion-abstracted fragment ions shifted by 1 Da from those in the corresponding EI mass spectra [22]. Using ammonia as reagent gas intense ammonia-molecular ion adducts of the methyl esters, with little, or no secondary fragmentation were reported for the detection of NPECs [26]. Ions selected were as follows: *m/z* 246, 310, 354 and 398 for NPE_1C, NPE_2C, NPE_3C and NPE_4C, respectively.

3. Elimination by activated sludge treatment (AST)

The present state-of-the-art of wastewater treatment involves treatment by the activated sludge treatment (AST) process proceeded with conventional physico-chemical pre-treatment steps. Table 3. summarizes data on the elimination of emerging contaminants in WWTP.

3.1. Pharmaceuticals and personal care products

Daughton and Ternes [1] reviewed the occurrence of over 50 individual PPCPs, or metabolites from more than 10 broad classes of therapeutic agents, or personal care products in environmental samples, mainly in WWTP effluents, surface, and ground waters and much less frequently in drinking waters. Acidic drugs are the major group of PPCPs detected in municipal WWTP and among them bezafibrate, naproxen, and ibuprofen were the most abundant (concentrations up to 4.6 μg/l were detected in German municipal WWTPs). Tixier et al. [27] determined that carbamezapine presented the highest daily load

from the WWTP into Lake Greifensee (Switzerland), followed by diclofenac, and naproxen. Their elimination during passage through a municipal sewage treatment in most cases was found to be quite low (see table 3), ranging from 35 to 90% and some compounds, like carbamazepine, exhibit extremely low removal (only 7%) [28]. Consequently, through sewage effluents they can enter receiving surface waters and thus become a potential risk in the production of drinking water. For example, the clofibric acid, a metabolite of three lipid regulating agents (clofibrate, etofibrate and fenofibrate) has been identified in river and ground water and even in drinking water at concentrations ranging up to 165 ng/l [29,30].

3.2. Acidic Pesticides

Chlorinated phenoxyacids are a kind of compounds widely used in agriculture. This group includes, for example, mecoprop (MCPP), MCPA, 2,4-D, 2,4-DP 2,4,5-T, 2,4-DB. Monitoring of these herbicides is important in surface water because of their potential toxicity towards animals and humans [31], however, these compounds are not only used for agricultural purposes, but also as herbicides on lawns, algicides in paints and coatings or as roof protection agents in flat roof sealings. So, residues of these substances are introduced into the aquatic system through different pathways. For example, in the catchment area of Lake Greifensee (Switzerland) 65% of the mecoprop originated from WWTPs and the remaining 35% from diffuse sources [32].

Degradation of acidic pesticides under laboratory conditions is well studied, but there are only few publications dealing with their behaviour in real wastewater treatment processes. Generally, activated sludge treatment was found to be ineffective in removing chlorinated phenoxy acid herbicides from settled sewage. However, under laboratory

conditions mecoprop proved to be biodegradable (nearly 100%), however it required long adaptation time (lag-phase) of activated sludge [33]. In real wastewater treatment processes this presents a major difficulty since, like the majority of herbicides, mecoprop is applied only during a short growth period of plants, which means that during this period WWTPs, that contains a non-adapted activated sludge, receive shock-loads of herbicides and consequently these substances are not eliminated.

A long acclimation period (about 4 months) was also observed in a bench-scale study using sequencing batch reactors before 2,4-D biodegradation was established [34]. Afterwards, at steady-state operation, all reactors achieved practically complete removal (>99%) of 2,4-D.

3.3. Alkylphenolic surfactants

Although their environmental acceptability is strongly disputed APEOs are still among the most widely used non-ionic surfactants. Currently, under optimised conditions, more than 90-95% of surfactants are eliminated by conventional biological wastewater treatment (normally activated sludge treatment). Even if such high elimination rates are achieved, the principal problem is the formation of recalcitrant metabolites out of the parent surfactants. The widespread occurrence of APEO-derived compounds in treated wastewaters and the following disposal of effluents into aquatic system raise concerns about their impact on the environment. Studies have shown that their neutral (alkylphenols and short ethoxy chain ethoxylates) and acidic recalcitrant metabolites (APEC) possess the ability to mimic natural hormones by interacting with the estrogen receptor.

It was estimated that approximately 60 to 65% of all nonylphenolic compounds introduced to WWTP are discharged into the environment; 19% in the form of

carboxylated derivatives, 11% in the form of lipophilic NP_1EO and NP_2EO, 25% in the form of NP and 8% as untransformed NPEOs [35].

However, contrary to the general believing that NPECs are the refractory metabolites, Di Corcia et al. [36] determined that CAPECs are the dominant products of the NPEO biotransformation. By averaging data relative to the treated effluents of five major activated sludge WWTP of Rome (Italy) over 4 months, relative abundances of NPEO (n_{EO}=1 and 2), NPECs and CAPECs were found to be respectively 10±2%, 24±5% and 66±7%.

The average concentrations of acidic metabolites, NPECs and CAPECs are at low μg/L range, however high values, up to several tens of hundreds of μg/L, are detected in effluents of WWTP receiving industrial wastewaters, especially from tannery, textile, pulp and paper industry [37].

4. Elimination by advance wastewater treatment processes

Although, adopted as the best available technology; biological treatment permits only partial removal of a wide range of emerging contaminants, especially polar ones, which are discharge into the final effluent. Thus, it has become evident that application of more enhanced technologies may be crucial for the fulfilment of the requirements of an indirect potable reuse of municipal and industrial wastewater. In recent years, new technologies are being studied, not only for wastewater treatment but also for drinking water production. Among them membrane treatment, using both biological (membrane bioreactors) and non-biological processes (reversed osmosis, ultrafiltration, nanofiltration),

and advanced oxidation processes (AOP) are most frequently considered as treatments that may be appropriate to remove trace concentrations of polar emerging contaminants.

4.1. Membrane processes

Membrane bioreactor (MBR) technology is considered as the most promising development in microbiological wastewater treatment. Now, when economic reasons do no longer limit the application of MBR to industrial and municipal wastewater (WW) treatment [38] and that new requirements are being set for the treatment of WW, MBR treatment may become a key technique in all future scenarios that consider the direct or indirect reuse of wastewaters. This is due to two characteristics of MBRs, (a) the low sludge load in terms of BOD that can be expected to force bacteria to mineralise also poorly degradable organic compounds and (b) the high sludge age that gives the bacteria time to adapt to these substances [39,40].

However, although many articles have reported the application of MBR for the treatment of urban and industrial wastewaters, up to our knowledge there are only few papers reporting on the behaviour of emerging contaminants during the MBR treatment, and all of them dealt with nonylphenolic compounds.

Using the MBR unit that comprises of three bioreactors and an external ultrafiltration unit followed by GAC adsorption, Witgens et al. [41] reported on the removal of more than 90% of NP in wastewater from a dumpsite leachate plant. Laboratory set of nanofiltration membranes resulted in retention of more than 70% of NP and this process was regarded as an alternative option for the final treatment of MBR effluent.

Li et al. [42] used GC-MS and LC-MS-MS to assess the elimination efficiency in membrane-assisted biological WWTP. The results showed that compared to conventional WWTP membrane assisted biological treatment with biomass concentrations of about 20g/l could only improve elimination efficiency of NPEOs (and other ionic and non-ionic surfactants), but could not stop entirely the discharge with the permeates.

4.2. Treatment by advanced oxidation processes (AOP)

The AOP processes, using the combination of ozone with other oxidant agents (UV radiation, hydrogen peroxide, TiO_2) have been studied to enhance the degradation of polar pharmaceuticals [43,44,45] and NPEOs metabolites [46]. Ternes et al. [45] used a pilot plant for ozonation and UV disinfection of effluents form a German municipal WWTP containing antibiotics, betablockers, antiphlogistics, lipid regulator metabolites, musk fragrances and iodinated X-ray contrast media. By applying 10-15 mg/l ozone (contact time 18 min) all the pharmaceuticals investigated were no longer detected. Exception was the ionic iodinated X-ray contrast compounds that exhibited removal efficiencies of not higher than 14%.

In another study [44] the ozonation has been demonstrated to be a suitable tool for carbamazepine abatment even at the process conditions usually adopted in drinking water facilities. However, in spite of good primary elimination, a low degree of mineralization was observed and total carbon balance results lacking even for prolonged ozonation thus indicative the presence of some non-identified degradation products.

However, the degradation efficiency of an AOP is limited by the radical scavenging capacity of the matrix of the treated water. Thus, for a sufficient degradation of the

pharmaceuticals (>90%) from wastewater the ozone concentration has to be equal to the dissolved organic carbon (DOC) value [43], which means that economic considerations have to prove the feasibility of the process for wastewater treatment.

Recently, using a lab-scale reactor, Ike et al. [46] determined that the effectiveness of ozone treatment in the degradation of NPEO metabolites follows the order: NPE_1C>>NP>NP_1EO. Acidic metabolites were completely degraded within 4-6 min (initial concentration was between 0.4 and 1.0 mg/l), NP concentration reduced 75-80% in 6 min, while only 25 to 50% of NP_1EO was eliminated in the same time.

5. Elimination by treatment processes at drinking water treatment plants

The occurrence of organic micro-contaminants in raw water and their removal in the course of drinking water production and possible formation of disinfection by-products are key issues in relation to the quality of drinking water supplies. Although, substances covered by this review are currently not regulated in drinking water, precautionary principles should be employed, and the removal of all organic micro-contaminants should be as high as possible. However, several studies showed that the removal of polar emerging contaminants during the drinking water treatment is not complete.

The elimination of selected pharmaceuticals (clofibric acid, diclofenac, carbamezapine, bezafibrate) during drinking water treatment processes was investigated at lab and pilot scale and in real waterworks in Germany [47]. Sand filtration under aerobic and anoxic conditions, as well as flocculation using iron(III)chloride exhibited no significant elimination of the target pharmaceuticals, while ozonation was quite effective in

eliminating these polar compounds. Diclofenac and carbamezapine were reduced by more than 90%, bezafibrate was eliminated by 50%, while clofibric acid was stable even at high ozone doze. Filtration with granular activated carbon (GAC), under waterworks conditions was very effective in removing pharmaceuticals. Exception was clofibric acid which was less prone to adsorption.

The behaviour of polar alkylphenolic compounds during processing of contaminated water in waterworks and their possible occurrence in treated water has been was rarely the scope of interest and there are hardly any data available for drinking water. The elimination of neutral and acidic nonylphenolic compounds and their brominated and chlorinated derivatives, during drinking water treatment process at the waterworks that supply drinking water to city of Barcelona (Spain) was investigated utilizing a highly sensitive LC-MS-MS method [48]. The concentration of total nonylphenolic compounds: NPEC (n_{EO}=0-1), NPEO (n_{EO}=1-2) and NP; in raw water (the Llobregat river) entering waterworks ranged from 8.3 to 21.6 μg/L, with NPE_2C being the most abundant compound. Prechlorination reduced the concentration of short-ethoxy chain NPECs and NPEOs by about 25-35%, and of NP by almost 90%. However, this reduction of concentrations was partially due to their transformation to halogenated derivatives. After prechlorination halogenated nonylphenolic compounds represented approximately 13% of the total metabolite pool, of which 97% were in the form of brominated acidic metabolites. The efficiency of further treatment steps to eliminate nonylphenolic compounds (calculated for the sum of all short ethoxy chain metabolites including halogenated derivatives) was as follows: settling and flocculation followed by rapid sand filtration (7.3%), ozonation (86.3 %), GAC filtration (72.7%) and final disinfection with chlorine (42.8%), resulting in overall elimination ranging from 96.2 to 99.1% (mean 97.9% for four sampling dates) as shown in Fig. 2.

6. Conclusions

The application of advanced LC-MS and GC-MS technologies to environmental analysis has allowed the determination of a broader range of compounds and thus permitted more comprehensive assessment of environmental contaminants. Among the various compounds considered as emerging pollutants, acidic pharmaceuticals, surfactant degradates and acidic pesticides are of particular concern, both because of their ubiquity in the aquatic environment and potential impacts.

Elimination of these emerging contaminants during wastewater and drinking water treatment is not satisfactory; and an improved treatment and strict control of the treatment process have to be employed so that the removal of these micro-contaminants is as high as possible. Thus, in view of possible reuse of WWTP effluents, more research is needed to evaluate their behaviour and fate in the aquatic environment. Moreover, disinfection processes applied (either chlorination or ozonation) potentially shift the assessment of the risk of human consumption of the parent compound to its degradation products, which requires development of generic analytical protocols that will permit simultaneous determination of parent compounds and their metabolites.

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References

(after tables and figures)

Figure captions

Fig. 1. Components of a (partially) closed water cycle with indirect potable reuse

Fig. 2. Fate of nonylphenolic compounds during drinking water production. A) Total concentration of nonylphenolic compounds and their elimination during different treatment steps at waterworks Sant Joan Despì (Barcelona, Spain); B) Average composition (calculated on a molar basis) of nonylphenolic compounds in raw water; C) Average composition (calculated on a molar basis) of nonylphenolic compounds in prechlorinated water

Table 1. Methods for the analysis of acidic pharmaceuticals in wastewaters

Compounds	Extraction	Derivatization	Chromatographic method	Detection	LOD (ng/L)	Reference
Bezafibrate, diclofenac, ibuprofen, gemfibrozil, carbamezapine	Sequential SPE (C18 + polymeric sorbent)	-	LC	MS	2	49
Salicylic acid, ibuprofen, ketoprofen, naproxen, bezafibrate, diclofenac	SPE (polymeric sorbent)	-	LC	MS	5-56	5
Bezafibrate, clofibric acid, diclofenac, fenoprofen, gemfibrozil, ibuprofen, inomethacin, ketoprofen, naproxen	SPE (C18)	-	LC	MS-MS	5-20	50
Bezafibrate, clofibric acid, ibuprofen	SPE (MCX or polymeric sorbent)	-	LC	MS-MS	0.016-2.18	51
Ibuprofen, clofibric acid, ketoprofen, naproxen, diclofenac	SPE (HLB)	diazomethane	GC	MS	0.3-4.5	52
Clofibric acid, diclofenac, ibuprofen, phenazone, propyphenazone	SPE (C18)	Pentaflorobenzyl bromide	GC	MS	0.6-20	53
Clofibric acid, naproxen, ibuprofen	SPE (polar Empore disk)	BSTFA (bis (trimethylsilyl)-triflouroacetamide	GC	MS	0.4-2.6	54
Ibuprofen, naproxen, ketoprofen, tolfenamic acid, diclofenac, meclofenamic acid	SPE (HLB)	MTBSTFA (N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide	GC	MS	20	55

Table 2. Quantitation and diagnostic ions (*m/z*) used for the LC-MS and GC-MS, and base peaks of precursor and product ions used for LC-MS-MS analysis of acidic pharmaceuticals in wastewaters. Data compiled from references listed in Table 1.

Compound	Analytical method	Ionization mode	MS	MS-MS		
			SIM ions	Precursor (*m/z*)	Product 1 (*m/z*)	Product 2 (*m/z*)
Ibuprofen	LC-MS	NI	205, 159			
	LC-MS-MS	NI		205 $[M–H]^–$	161 $[M–H–CO_2]^–$	-
	GC-MS	Positive EI	177, 220[a]			
			161, 343, 386[b]			
			263, 278, 234[c]			
Diclofenac	LC-MS	NI	294, 250, 232			
	LC-MS-MS	NI		294 $[M–H]^–$	250 $[M–H–CO_2]^–$	-
	GC-MS	Positive EI	214, 309[a]			
			214, 216, 475[b]			
			352/354/356[d]			
Clofibric acid	LC-MS	NI				
	LC-MS-MS	NI		213 $[M–H]^–$	127 $[C_6H_4ClO]^–$	85 $[C_4H_5O_2]^–$
	GC-MS	Positive EI	128, 228[a]			
			128, 130, 394[b]			
			128, 143, 286[c]			
Benzafibrate	LC-MS	NI	360, 274			
	LC-MS-MS	NI		360 $[M–H]^–$	274 $[M\text{-}H\text{-}C_4H_6O_2]^–$	154 $[M\text{-}H\text{-}C_{12}H_{14}O_3]^–$
	GC-MS	Positive EI	128, 228[a]			
			128, 130, 394[b]			
Gemfibrozil	LC-MS	NI	249, 121			
	LC-MS-MS	NI		249 $[M–H]^–$	121 $[M\text{-}H\text{-}C_7H_{12}O_2]^–$	
Ketoprofen	LC-MS	NI	253, 209, 197			
	LC-MS-MS	NI		253 $[M–H]^–$	209 $[M\text{-}H\text{-}CO_2]^–$	
	GC-MS	Positive EI	209, 268[a]			
			311[d]			
Naproxen	LC-MS	NI	229, 185, 173, 170			
	LC-MS-MS	NI		229 $[M–H]^–$	185 $[M\text{-}H\text{-}CO_2]^–$	170 $[M\text{-}H\text{-}C_2H_3O_2]^–$
	GC-MS	Positive EI	185, 244[a]			
			243, 302, 185[c]			
			287[d]			

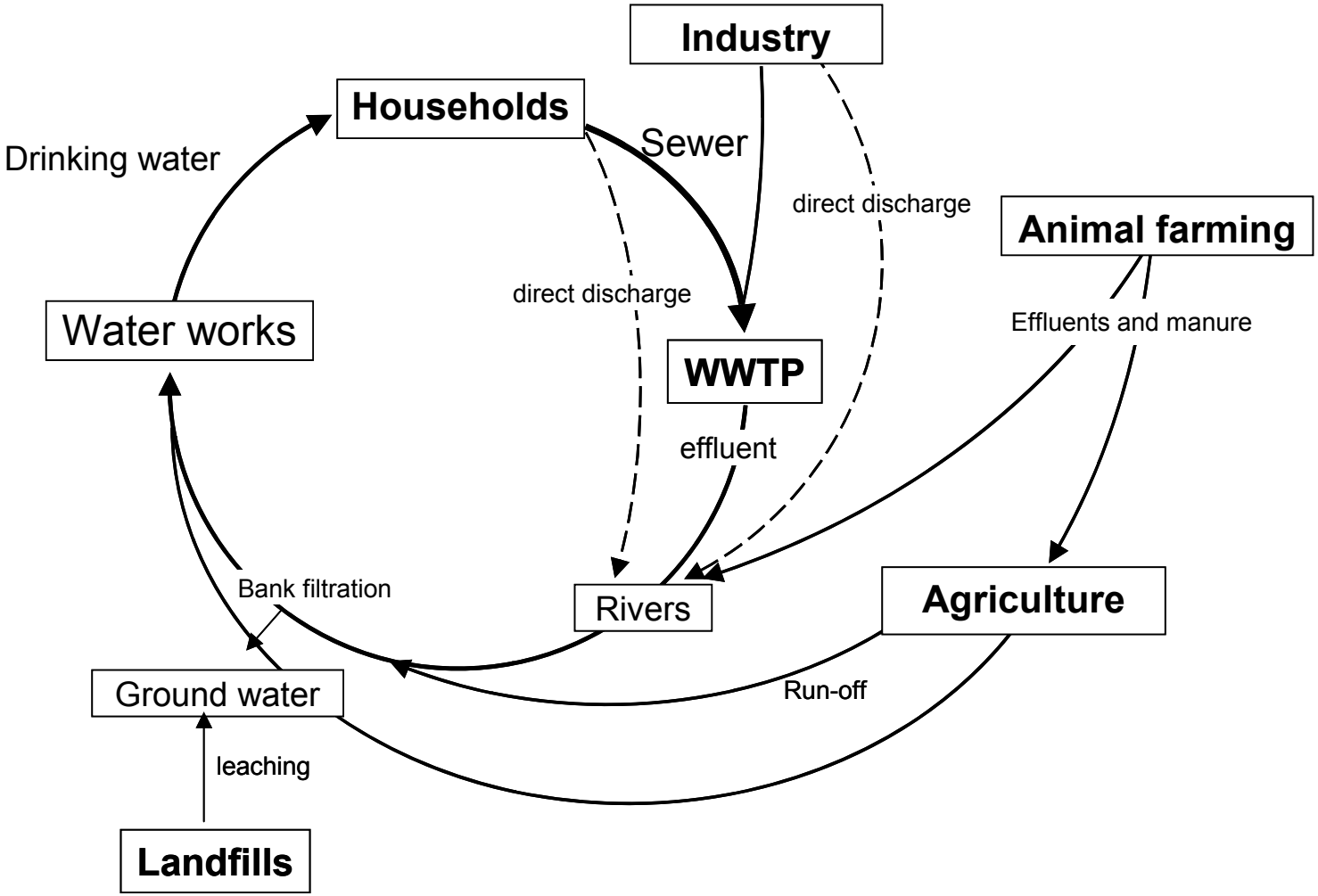
[a] diazomethane derivative, [b] pentaflourobenzyl derivative, [c] trimethylsilyl derivative, [d] tert-butyldimethylsilyl derivative

Table 3. Elimination at WWTP (activated sludge treatment). Data complied from references [12,28,37,52,55,56,57,58]

Compound	Average elimination (%)[a]	Effluent concentrations (μg/L)	Main degradation products	Observation
Non ionic surfactants				
Alkylphenol ethoxylates	90– 99	<0.1–350	APEC, CAPEC, AP	Primary degradation fast; ultimate degradation less than 40%, metabolites potential endocrine disruptors
Pharmaceuticals				
Ibuprofen	65–90	0.37-0.60 (3.4) [b]		
Diclofenac	69–75	0.06-0.81 (2.1)		Rapid photodegradation
Clofibric Acid	34–51	0.12-0.36 (1.6)		Degradation product of lipid regulating agents
Benzafibrate	83	1.1-2.2 (4.6)		
Naproxen	45–66	0.27-0.61 (2.6)		
Ketoprofen	69	0.02-0.38 (0.87)		
Gemfibrozil	46–69	0.31-0.40 (1.9)		
Carbamazepine	7	0.30-2.1 (6.3)		Low removal rate
Antiseptics				
Triclosan	44–92	0.070–0.650	Methyl triclosan	Possible photodegradation
Pesticides				
Mecoprop and MCPA	-	20–400	2-methyl-4-Cl-phenol	Application period (mid-March until mid-May)
2,4-D	-	<20	2,4-dichlorphenol	
2,4,5-T	-	<20	2,4-D ; 2,4-dichlorphenol	

[a] Primary elimination of the parent compound

[b] Range of average values detected (in parenthesis: maximum concentration detected)


Industry
Households
Sewer
Drinking water
direct discharge
Animal farming
direct discharge
Water works
Effluents and manure
WWTP
effluent
Bank filtration
Rivers
Agriculture
Ground water
Run-off
leaching
Landfills

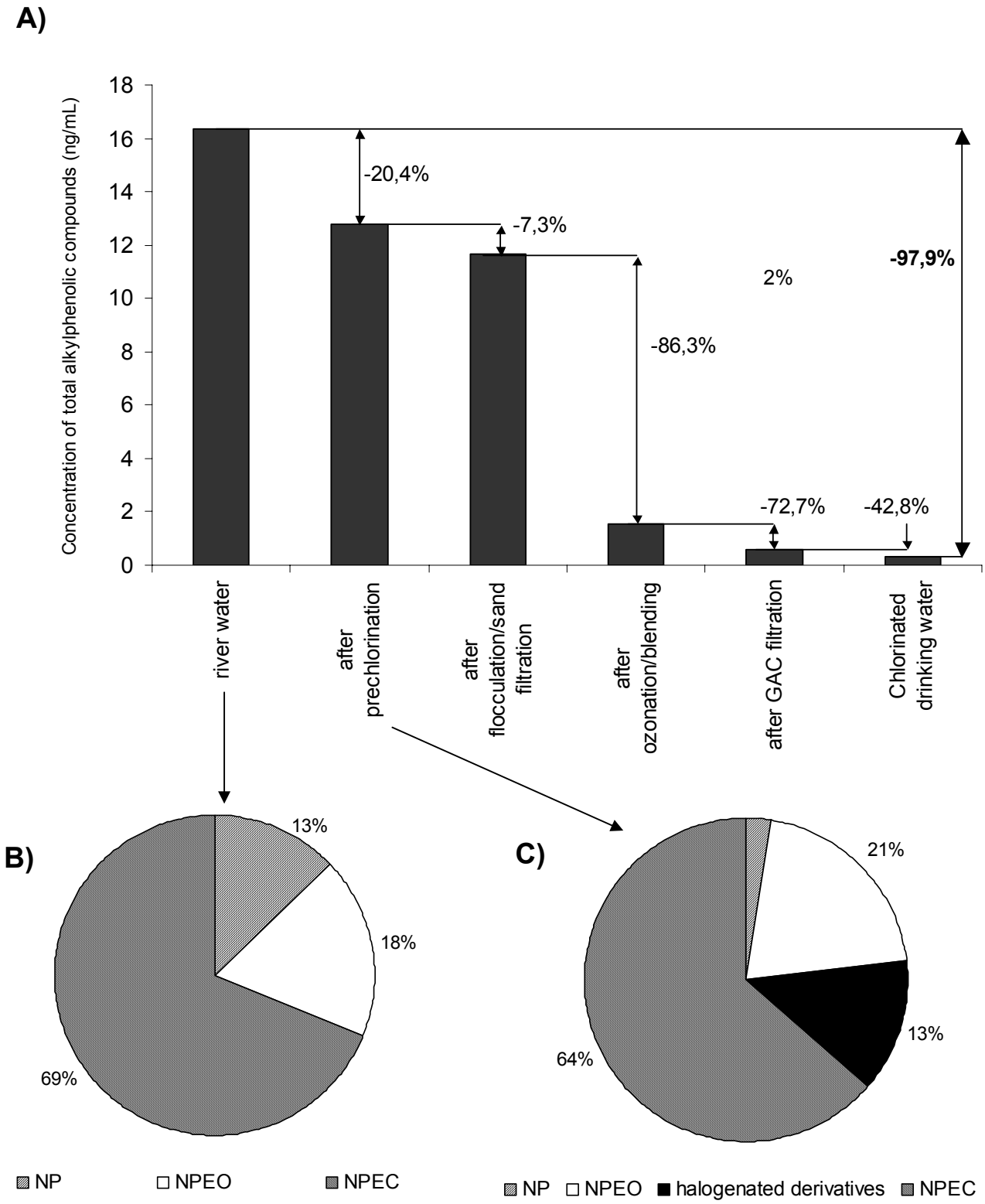

A)
Concentration of total alkylphenolic compounds (ng/mL)
18
16
14
12
10
8
6
4
2
0
-20,4%
-7,3%
-86,3%
-72,7%
-42,8%
2%
-97,9%
river water
after prechlorination
after flocculation/sand filtration
after ozonation/blending
after GAC filtration
Chlorinated drinking water
B)
13%
18%
69%
NP
NPEO
NPEC
C)
21%
13%
64%
NP
NPEO
halogenated derivatives
NPEC

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Exhibit 4

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Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado†

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This study explores antibiotic resistance genes (ARGs) as emerging environmental contaminants. The purpose of this study was to investigate the occurrence of ARGs in various environmental compartments in northern Colorado, including Cache La Poudre (Poudre) River sediments, irrigation ditches, dairy lagoons, and the effluents of wastewater recycling and drinking water treatment plants. Additionally, ARG concentrations in the Poudre River sediments were analyzed at three time points at five sites with varying levels of urban/agricultural impact and compared with two previously published time points. It was expected that ARG concentrations would be significantly higher in environments directly impacted by urban/agricultural activity than in pristine and lesser-impacted environments. Polymerase chain reaction (PCR) detection assays were applied to detect the presence/absence of several tetracycline and sulfonamide ARGs. Quantitative real-time PCR was used to further quantify two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)). The following trend was observed with respect to ARG concentrations (normalized to eubacterial 16S rRNA genes): dairy lagoon water > irrigation ditch water > urban/agriculturally impacted river sediments ($p < 0.0001$), except for sul(II), which was absent in ditch water. It was noted that tet(W) and tet(O) were also present in treated drinking water and recycled wastewater, suggesting that these are potential pathways for the spread of ARGs to and from humans. On the basis of this study, there is a need for environmental scientists and engineers to help address the issue of the spread of ARGs in the environment.

Introduction

The spread of antibiotic-resistant pathogens is a growing problem in the U. S. and around the world. Recently a 2000 World Health Organization (WHO) report (*1*) focused on antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance". According to the report, more than two million Americans are infected each year with resistant pathogens and 14 000 die as a result. The rapid growth of the problem emphasizes the need for intervention. For example, vancomycin is currently considered to be the most powerful antibiotic of "last resort", yet within 10 years the incidence of vancomycin-resistant enterococci (VRE) increased in the United States from 0% to 25% (*2*, *3*). Resistance to penicillin, the antibiotic that originally revolutionized human health 50 years ago, is now as high as 79% in *Staphylococcus pneumoniae* isolates in South Africa (*4*, *5*). Alarmingly, diseases that were once considered to be eradicated, such as tuberculosis, are now beginning to make a comeback because of antimicrobial resistance (*1*, *6*, *7*). As with other dangerous pollutants that spread in the environment and threaten human health, there is a need for environmental scientists and engineers to help address the critical problem of microbial resistance to antibiotics.

The rise of antibiotic resistance is considered to be closely linked with the widespread use of antibiotic pharmaceuticals in humans and animals. In particular, more than one-half of the antibiotics used in the U. S. are administered to livestock for purposes of growth promotion or infection treatment (*8*, *9*). In both animals and humans, up to 95% of antibiotics can be excreted in an unaltered state (*10*, *11*). Some removal has been observed in wastewater treatment plants (WWTPs); however, as is true with the larger problem of pharmaceutical compounds, WWTPs are not designed for the removal of micropollutants (*12*−*14*). Residual antibiotics thus are released into the environment where they may exert selection pressure on microorganisms. While overprescribing or other improper use/disposal of antibiotics in humans is generally considered to contribute to the problem, several studies have also linked agricultural antibiotic use with antibiotic-resistant infections in humans (*15*−*23*). For example, avoparcin, an antibiotic growth-promoter used in poultry, was recently banned in Europe because of its association with the development of vancomycin-resistant enterococci (*24*).

Because of the direct selection pressure that antibiotics exert on organisms carrying antibiotic resistance genes (ARGs), the transport pathways of antibiotic-resistant microorganisms and the ARGs that they carry are expected to be similar to the pathways of antibiotic pharmaceuticals. In fact, it is likely that ARGs persist further in the pathway, considering that in many cases they are maintained in the microbial populations even after the antibiotic selection pressure has been removed (*25*−*28*). Also, horizontal gene transfer (HGT) is a major mechanism for sharing ARGs between microbes and has been documented to occur between nonpathogens, pathogens, and even distantly related organisms, such as Gram-positive and Gram-negative bacteria (*25, 29*−*31*). In many cases, ARGs have been discovered to occur as part of multiple antibiotic resistant (MAR) superintegrons, which may contain over 100 ARG cassettes (*32*). These MAR superintegrons cause multiple-drug resistance in organisms, meaning that even when very different antibiotics are used, one antibiotic may coselect for resistance to other antibiotics (*5*, *33*). MAR gene cassettes and ARGs are notorious for being associated with plasmids and/or transposons that facilitate HGT. Finally, even if cells carrying ARGs have been killed, DNA released to the environment has been observed to persist, to be protected from DNAse, especially by certain soil/clay compositions, and to be eventually transformed into other cells (*34*−*36*). For all of these reasons, ARGs in and of themselves can be considered to be emerging "contaminants" for which mitigation strategies are needed to prevent their widespread dissemination.

The purpose of this study was to document the occurrence of tetracycline and sulfonamide ARGs in various environmental compartments in northern Colorado. These two ARG

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groups were chosen because sulfonamide and tetracycline antibiotics have been previously characterized in Poudre River sediments and shown to relate to urban/agricultural activity (*37*). The breadth of the study included Cache La Poudre (Poudre) River sediments, dairy lagoon water, irrigation ditch water, a wastewater recycling plant (WRP), and two drinking water treatment plants (DWTPs). The hypothesis was that environmental compartments most directly impacted by urban/agricultural activity would have significantly higher concentrations of ARGs than less impacted and pristine environments. Irrigation ditch waters, which were directly adjacent to farms, were investigated as a potential pathway of ARGs from farms to the Poudre River, while the WRP and the DWTPs were explored as potential routes of human environmental input and consumption. The presence/absence of several ribosomal protection factor tetracycline ARGs and folic acid pathway sulfonamide ARGs was determined using a polymerase chain reaction (PCR) detection assay, and four commonly occurring ARGs were further quantified by quantitative real-time PCR (Q-PCR). Documenting the baseline occurrence of ARGs in a cross-section of environmental compartments will take a step toward understanding and modeling the fate and transport phenomena associated with these emerging contaminants.

Experimental Section

Poudre River Sediment Sampling. Because of its pristine origins and zonation corresponding to land use, the Poudre River has served as a good model for relating human and agricultural activities with the occurrence of antibiotic pharmaceuticals (*37*) and ARGs (*38*). Five sampling sites were the focus of this study, numbered sequentially in the direction of flow from west to east, with the following characteristics: site 1, pristine location at the river origin in the Rocky Mountains; site 2, light-agriculture-influenced area; site 3, urban-influenced area at the outlet of the Fort Collins Drake WWTP; site 4, heavy-agriculture-influenced area between Fort Collins and Greeley; and site 5, heavy-agriculture- and urban-influenced area just east of Greeley, which is a major center for the meat-packing industry. Over 90 confined animal feeding operations (CAFOs), dairies, and ranches are located between sites 3 and 5. Further attributes of the Poudre River watershed that contribute to its suitability for investigating the impacts of urban and agricultural activity on antibiotics and ARGs have been described previously (*37*, *38*).

Sediment samples were collected along the Poudre River at the five sites on August 18, 2005, October 27, 2005, and February 17, 2006. The flow rates on these three dates were 1.04, 14.19, and 0.14 $m^3\ s^{-1}$, respectively (U. S. Geological Survey station number 06752260, Fort Collins, CO). Sampling at three points in time provided insight into potential temporal variations in ARG concentrations, and the February 17th date is exactly 1 year later than a previously published sampling date (*38*). The upper sediments (about 5 cm) from the middle and two sides of a cross-section at each site were sampled and composited. Samples were collected using a shovel and mixed well in sterilized centrifuge tubes. Fifty-five grams of mixed sample at each site were stored at −80 °C for subsequent molecular analysis.

Bulk Water Sampling. Irrigation ditch waters were investigated as a potential pathway of ARGs from farms to the Poudre River. Grab samples of bulk water were collected in sterile containers from irrigation ditches on August 18, 2005, corresponding to the August sampling date of the Poudre River sediments. All irrigation ditches were located between site 4 and site 5 on the Poudre River within a 3.5 km × 2 km zone north of the river, and a total of ten locations were sampled. To investigate a potential source of ARGs within this zone, a microaerophillic dairy lagoon (~1 mg/L dissolved oxygen in the upper 1 m) and an anaerobic dairy lagoon (0 mg/L dissolved oxygen) from an anonymous farm located 8 km from site 5 were sampled on October 20, 2005. Finally, source water, and pre-chlorinated, and post-chlorinated bulk water were collected from two anonymous DWTPs and an anonymous WRP in northern Colorado in February, 2005. The DWTP was studied as a potential direct route of ARGs to consumers, and the WRP was considered a potential human input into the environment. To collect fine particulates from the dilute ditch water, DWTP, and WRP samples for subsequent analysis, 500 mL of well-mixed sample was filtered using a 0.45 μm glass fiber filter (Whatman). This concentration step was not required for dairy lagoon samples.

DNA Extraction. DNA was extracted from 0.5 g of composited sediment using the FastDNA Spin Kit for Soil (MP Biomedicals) and from 1.8 mL of dairy lagoon water using the Ultraclean Microbial DNA Kit (MoBio Laboratories, Inc.) according to manufacturer protocol. Both approaches employ a bead-beating procedure. For fine particulates collected on filters from bulk water, the filters were cut into small pieces and added directly to the extraction tubes. Extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry.

Detection and Quantification of ARGs. Polymerase chain reaction detection assays were used for broad-scale screening of the presence/absence of five ribosomal protection factor tetracycline ARGs (tet(BP), tet(O), tet(S), tet(T), and tet(W)) (*39*) and four folic acid pathway sulfonamide ARGs (sul(I), sul(II), sul(III), and sul(A)). Development and validation of sul primers was described in Pei et al. (*38*). Positive controls consisted of cloned and sequenced PCR amplicons obtained from Poudre River sediments. Both positive and negative controls were included in every run, and negative signals were confirmed by spiking positive control template into the sample to verify a signal. Forty cycles were used to improve chances of product formation from low initial template concentrations. Further details on reaction mixes and temperature programs are available in Pei et al. (*38*); note that annealing temperatures for tet primers vary from Aminov et al. (*39*). Two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)) that were commonly occurring according to the PCR presence/absence assays were further quantified by Q-PCR using a SybrGreen approach. For further details on Q-PCR methods, see Pei et al. (*38*). Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method described by Suzuki et al. (*40*) so that ARGs could be normalized to the total bacterial community. This provided a means to correct for potential variations in extraction efficiencies. By quantification of 16S rRNA genes, it was also possible to compare ARGs proportionally between samples of different overall population sizes. Matrix effects associated with extraction of DNA from environmental samples were corrected for by performing spiked matrix control tests and determining template suppression factors as described in Pei et al. (*38*). All Q-PCR analyses were performed using a Cepheid SmartCycler (Sunnyvale, CA).

Statistics. The influences of the environment (sites, ditch water, and dairy lagoons) on the normalized and non-normalized copies of ARGs were analyzed using the Mixed Procedure, which fits a variety of mixed linear models to data. This provides the flexibility of simultaneously modeling means, variances, and covariances (*41*−*44*). Through the use of this test, it was thus possible to comprehensively compare overall differences between different environmental compartments with respect to ARG concentrations. For comparison of the five Poudre River sites, multiple sampling time points were treated as replicates. Mixed Procedures were conducted using SAS 9.0 (SAS Institute Inc., Cary, NC). A

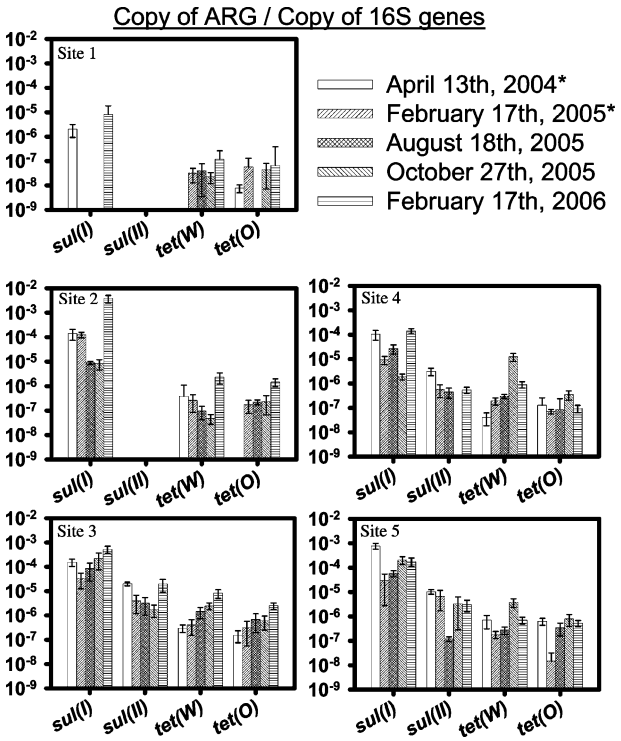

FIGURE 1. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet-(W)) in Poudre River sediments on three sampling dates, compared to two previously published sampling dates (April 13, 2004, and February 17, 2005 (*38*)), as determined by Q-PCR: site 1, pristine site; site 2, light agricultural activity; site 3, heavy urban activity; site 4, heavy agricultural activity; site 5, heavy urban and agricultural activity. Error bars represent the standard deviation of six measurements from three independent Q-PCR runs analyzing DNA extract from composite samples.

p-value <0.05 was considered to indicate significance. Averages and standard deviations of all data were determined using Microsoft Excel, 2003.

Results and Discussion

Occurrence of ARGs in Northern Colorado. Figure 1 summarizes the Q-PCR data obtained for the four ARGs at the five Poudre River sites, while Figure 2 summarizes the same analyses for the ditch waters and dairy lagoon water. When August 2005 data for the Poudre River sediments are compared with the dairy lagoon and ditch water, the following trend is observed with respect to ARG concentrations: dairy lagoon water > ditch water > river sediments ($p < 0.0001$), for all ARGs except sul(II), which was absent from the ditch waters. This is based on pooling of all 10 ditch water sites, the two dairy lagoons, and sites 4 and 5, which were directly adjacent to the ditch water sampling locations. Within each of these three pools, there was no statistical difference observed among the samples. Therefore, it was observed as expected that environmental compartments most directly impacted by human/agricultural activity showed higher concentrations of ARGs. This trend is even stronger in considering absolute quantities of ARGs (not normalized to 16S rRNA genes), because the concentration of cells in the dairy lagoon water was orders of magnitude higher than that of the ditch water or the sediments.

In developing a hypothetical pathway for ARGs, a trend is not as clear. The overall trend in terms of ARG concentrations of dairy lagoon water > ditch water > river sediments suggests that on-farm compartments, such as lagoons may be the source of ARGs, which are subsequently attenuated in ditch water before reaching Poudre River sediments.

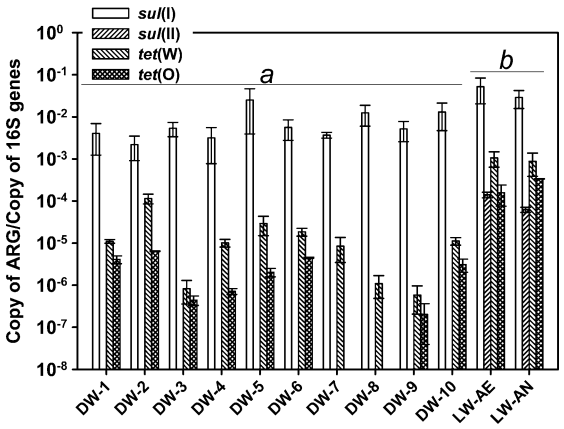

FIGURE 2. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet-(W)) at 10 sampling points of irrigation ditch water (DW-1−DW-10) located between site 4 and site 5 compared with that of a microaerophillic dairy lagoon (LW-AE) and an anaerobic dairy lagoon (LW-AN). DW samples were concentrated from 500 mL, and LW samples were extracted directly from 1.8 mL. All samples were normalized to the total 16S rRNA genes. Error bars represent three independent Q-PCR runs in duplicate. The labels a and b indicate that the data sets fell into two statistically different groups, according to the Mixed Procedure.

However, this trend is not supported in terms of sul(II), which is entirely absent from the ditch water and therefore cannot be the source of what is observed in the Poudre River sediments. An alternative source of the sul(II) that appears at sites 4 and 5 could instead be human inputs. This is supported by the data presented in Figure 1, in which it is observed that sul(II) is consistently present at high levels on average at site 3, which is at the point of discharge of the Drake WWTP, while consistently lower (comparing each date sampled) at site 4 (entirely absent for the October event) and equivalent or lower at site 5, which has mixed human/agricultural inputs. Because sul(II) is present in the dairy lagoon waters, it must also have agricultural sources, but it may attenuate too quickly to be transported to the ditches and subsequently to the river sediments. On the basis of this study and a previous study (*38*), it is appears that of the four ARGs quantified sul(II) is the most sensitive indicator of human/agricultural impact, and thus it is suggested that it attenuates quickly in the absence of direct inputs. The other ARGs in the Poudre River sediments at sites 4 and 5 may be of either/both human and agricultural origin, since they followed a decreasing trend from the dairy lagoon through the ditch water but were also present at site 3.

In addition to having higher concentrations of three out of four of the ARGs, the dairy lagoon water was also observed to have more different kinds of ARGs present than the irrigation ditch water according to the PCR assay (Table 1). Together with the Q-PCR results, these data further support the concept that there is some attenuation of ARGs between any linkages that may connect dairy lagoon water and irrigation ditch water. Future work should implement ARG fingerprinting/source tracking to fully characterize the potential pathways.

Temporal Variations of ARG in Poudre River Sediments. As observed in a previous study that compared a high-flow sampling point (6.8 m^3 s^{-1}, April 2004) with a low-flow sampling point (0.6 m^3 s^{-1}, February 2005), the ARG concentrations in the Poudre River sediments are variable with time (*38*). To better understand temporal variations in ARG concentrations, the Poudre River sediments were sampled at three additional time points and compared with the two previously published time points. The February sampling point in this study took place exactly 1 year after

TABLE 1. PCR Presence/Absence Assay of Various ARGs in Ditch (DW)[a] and Dairy Lagoon (LW) Water[b]

ARG	DW-1	DW-2	DW-3	DW-4	DW-5	DW-6	DW-7	DW-8	DW-9	DW-10	LW-AE	LW-AN	+ control
tet(BP)	−	−	−	−	−	−	−	−	−	−	−	−	+
tet(O)	+	+	+	+	+	+	−	−	+	+	+	+	+
tet(S)	−	−	−	−	−	−	−	−	−	−	−	−	+
tet(T)	−	−	−	−	−	−	−	−	−	−	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(II)	−	−	−	−	−	−	−	−	−	−	+	+	+
sul(III)	−	−	+	+	+	−	−	−	−	−	+	+	+
sul(A)	−	−	−	−	−	−	−	−	−	−	−	−	+

[a] Collected August 18, 2005. [b] Collected October 20, 2005.

the previous February event. In support of the relationship between ARG concentration and relative environment impact observed above, the pristine site (site 1) consistently had the lowest average concentrations of ARGs with time, with sul(II) completely absent and no individual ARG consistently present at all five sampling times (Figure 1). When presence/absence of ARGs are compared, site 2 appears to be the next lowest in terms of overall impacts. For example, sul(II) is consistently absent at site 2, and tet(O) was absent in one of the five sampling events, whereas these genes were consistently present at sites 3, 4, and 5. In terms of ARG concentrations, tet(W) and tet(O) at site 2 were equal or less than site 3; however, these two genes were sometimes higher and sometimes lower than at sites 4 and 5. On the basis of ARG averages and presence/absence of ARGs, sites 1 and 2 were the least impacted, as expected.

When the Mixed Procedure was applied to the data, in which the time points were pooled as replicates, it was found that there was no statistical difference between the five sites for the 16S normalized data, except in the case of sul(II) ($p = 0.0117$). However, when the same test was performed with non-normalized data, it was found that sites 1 and 2 were statistically lower than sites 3, 4, and 5 in terms of sul(I) ($p = 0.00296$), sul(II) ($p = 0.0199$), and tet(O) ($p = 0.0102$). Though normalizing to 16S genes provides a comparison of ARGs as a proportion of the total population, arguably it may be the absolute quantities of ARGs that are more critical.

While spatial variations in ARGs could be fairly well-characterized, it is difficult to identify clear temporal patterns. Comparison of the two February sampling dates that were exactly a year apart provides some insight. All four genes were either the same on average for both events (tet(O) for sites 1 and 4 and sul(II) for sites 4 and 5) or higher in the 2006 event (all other genes, except sul(II) at sites 1 and 2, where it was not present) (Figure 1). This suggests the possibility that all ARGs are increasing in concentration with time. However, the trends in between these two dates do not support this. Only tet(W) and tet(O) at site 3 increase consistently with time. All remaining ARGs at the five sites either decrease before increasing (e.g., tet(W) at site 2 and sul(II) at site 3), are constant and then increase (e.g., tet(O) at site 2 and tet(W) at site 1), or increase and then decrease (e.g., tet(W) at sites 4 and 5) (Figure 1). Therefore, no clear trend was identified with time.

It was also attempted to analyze trends in the data with respect to river flow rate. This was of interest because flow rate directly relates to runoff and nonpoint source inputs, which were hypothesized in the previous study to play a role in the observed increase in the number of kinds of ARGs detected in Poudre River sediments (*38*). The October 2005 sampling date provided a second sampling date at high flow (14.9 $m^3 s^{-1}$), compared to the previously published April 2004 high-flow sampling date (6.8 $m^3 s^{-1}$). (All other dates were at or below 1.0 $m^3 s^{-1}$.) Interestingly, all four ARGs increased on average at site 5 in comparing the high-flow

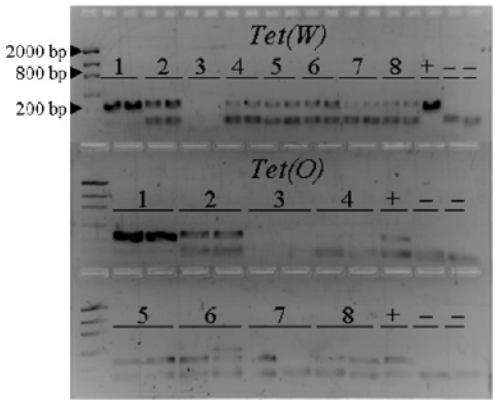

FIGURE 3. Agarose gel analysis of PCR presence/absence (in duplicate) of two ARG families, tet(W) and tet(O): + = positive control; − = negative control. The presence of a band at the same molecular weight as + indicates the presence of an ARG: 1 = WRP effluent; 2 = WRP chlorinated effluent; 3 = DWTP a influent; 4 = DWTP a treated water pre-chlorination; 5 = DWTP a treated water post-chlorination; 6 = DWTP b influent water; 7 = DWTP b treated water pre-chlorination; 8 = DWTP b treated water post-chlorination. The band appearing below 200 bp is consistent with a primer dimer.

October event with the immediately previous low-flow event in August (Figure 1). At site 4, tet(W) and tet(O) increased, but sul(II) stayed the same, and sul(I) decreased. There was no effect at all at site 3, which is affected primarily by point discharge rather than runoff, site 2, or site 1. However, attempts to plot ARG concentrations versus flow rate did not reveal any clear trend. Thus, it is still not possible to make a conclusive judgment on the effect of flow rate on ARG concentrations, though the role of nonpoint source inputs merits further investigation. To accomplish this, it would be necessary to gather more data with time/flow or monitor a much more controlled and smaller-scale system.

Wastewater Recycling Plant and Drinking Water Treatment Plants. A PCR presence/absence assay was conducted on the influent, intermediate effluent, and final effluent of two drinking water treatment plants (DWTP "a" and DWTP "b") and the pre-chlorinated and chlorinated effluent of a WRP. It was observed that both tet(W) and tet(O) were present at detectable levels in all samples except the source water for DWTP "a" (Figure 3). This indicates that the same two genes that were common in various environmental compartments in northern Colorado are also present in treated recycled wastewater and bulk drinking water. These two genes also showed a response to the level of impact; e.g., they were highest in dairy lagoon water and ditch water and lowest on average at the pristine site. On the basis of the intensity of the signal, they were also higher in the recycled wastewater than in the drinking water, as would be expected. Though these two ARGs are not directly associated with any known human pathogens, they may be indicators of links

between human/agricultural activity and ARGs in drinking water. Considering that drinking water is a direct route to human consumers, this emphasizes the need to better understand the pathways by which ARGs are spread in the environment and potential ways that the spread of ARGs may be reduced. For example, vancomycin resistance genes were found in drinking water biofilms in a recent study (*45*). Considering that vancomycin is typically the antibiotic of last resort when all else fails, this underscores the need to address this issue before it is too late. One possibility may be to make simple modifications to wastewater and drinking water treatment plants to reduce the spread of ARGs.

ARGs as Emerging Contaminants. On the basis of this study it is clear that ARGs are present in various environmental compartments, including river sediments, irrigation ditch water, dairy lagoon water, DWTPs, and a WRP. Furthermore, quantitative techniques incorporating Q-PCR provide a means to compare the concentrations of ARGs associated with the known urban and agricultural impacts, which provides a more direct measure than previous culture-based methods. On the basis of this occurrence survey, it is argued that ARGs are emerging contaminants that need to be further studied in the paradigm of environmental science and engineering. The concept of ARGs as "pollutants" has also been suggested by Rysz and Alvarez (*46*).

It should be noted that besides the tetracycline and sulfonamide ARGs that were the focus of this study, there are numerous other ARGs that have been described in the literature and likely even more that have not yet been discovered, each potentially with its own unique properties. Thus, each ARG may have different behaviors with respect to fate and transport and response to physical, chemical, and/or biological treatment. In terms of defining fate and transport characteristics of ARGs in general, it is expected that their behavior will be distinct in comparison to "typical" contaminants. For example, ARGs may be sequestered with bacteria, which are themselves transported, or they may be present as naked DNA bound to clay particles (*47*). Furthermore, ARGs may actually amplify in the environment under some conditions. This is indeed a unique contaminant property. Considering the significance of the problem of the spread of antibiotic resistance, further effort by environmental researchers to better understand these emerging contaminants is well-warranted. This is especially true as the rate of discovery and development of new antibiotics is continually declining (*48*), while the corresponding development and spread of resistance is occurring at a rapid pace. On the basis of this study, understanding ARGs as emerging contaminants can add a new and important angle to helping to approach this important problem.

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