



**AXYS Analytical Services Ltd.**

**“EXPERIENCES in ANALYSIS of PPCPs  
and STEROIDS/HORMONES in POTW  
BIOSOLIDS”**

Richard Grace, Coreen Hamilton,  
Million Woudneh, Blair Surridge  
Brian Fowler

AXYS Analytical Services Ltd.  
Sidney, BC, Canada

**NELAP ACCREDITED**

**ISO 17025 CERTIFIED**



# AGENDA

- ❑ **Overview of Analytical Methods**
  - ❑ **PPCP Method**
  - ❑ **Hormone / Sterol Method**
- ❑ **Analytical Challenges**
  - ❑ **Clean Matrices vs. POTW Matrices**
  - ❑ **Positive Identification / False Positives and Suppression**
  - ❑ **Range of Analyte Concentration**
  - ❑ **Sample Handling / Preservation**



# PURPOSE of ANALYSIS

- ❑ **Ultra- Trace DL Targets (pg to ng)**
- ❑ **PPCP Method**
  - ❑ **Tool to Investigate Levels and Occurrence of PPCPs in Environment**
  - ❑ **73 Targets**
- ❑ **HORMONE / STEROL Analysis**
  - ❑ **Tool to Investigate Levels and Occurrence of Hormones and Sterols in Environment**
  - ❑ **27 Targets**
  - ❑ **Source Info / Fingerprinting**



# COMMON to BOTH ANALYSIS

- ❑ **Developed as per EPA 1600 Series Methods**
  - ❑ Performance based
  - ❑ Multi Matrix purpose and validation
  - ❑ Validated by Federal Register 40 CFR, Part 136, Appendix B
- ❑ **Utilize labeled surrogate addition at start of process, recovery corrected vs. surrogates**
- ❑ **Validated for Positive Identification as part of large analyte panel**
- ❑ **Focus on native compounds, not degradation products or metabolites**



# Rationale for Analyte Selection

- ❑ **Current or Historic Use / Occurrence**
- ❑ **Availability of Standards and Appropriate Surrogates**
- ❑ **Capture by SPE Cartridge (PPCP)**
- ❑ **Performance in Derivatization Process (Hormones and Sterols)**
- ❑ **Analyte Stability (Matrix and Instrument)**
- ❑ **Appropriate Monitoring Ions**



# BATCH QC

- Max. Batch Size (including QC) is 20 samples (8 to 14 is more common)
- Each Batch contains:
  - method blank
  - duplicate
  - Bracketing Cal.
  - Instrument Blank
  - MS/MSD, SRM, CRM included by request and applicability



# OVERVIEW of PPCP ANALYSIS in POTW BIOSOLIDS

Sample – 2 X 1 gram wet solids  
Acidic Fraction to pH 2 + EDTA stabilizer, Basic Fraction to pH10

Respective Fractions – Add labeled Surrogates  
Acidic Fraction – 3X ultrasonic extraction with phosphate buffer/ACN  
Basic Fraction – 3x ultrasonic extraction with H<sub>2</sub>O/NH<sub>4</sub>OH

SPE Cartridge Cleanup (1 gram Waters Oasis HLB)  
SPE Steps – condition, load, rinse, elute, reduce, transfer to MeOH  
Add recovery standards

ANALYSIS by LC/MS/MS (x4)  
ACIDIC EXTRACT – Neg. Ionization, Tetracyclines, Pos. Ionization  
BASIC EXTRACT – Pos. Ionization

# PPCP Standards and Surrogates

- Non-labeled standards for each target compound
- Stable isotope labeled standards where ever possible used as internal standards
  - 17 labeled standards ( $^{13}\text{C}$ ,  $^2\text{D}$  or  $^{15}\text{N}$ ) for acidic ESI+compounds
  - Labeled ( $^{13}\text{C}$ ) thiabendazole and sulfamethazine for tetracyclines
  - Exact labeled analogue for all 6 Acidic ESI - compounds
  - Labeled ( $^2\text{D}$ ) albuterol and metformin for basic compounds
- Labeled injection standards ( $^{13}\text{C}$  Atrazine or 2,4,5-TPP) to quantify recovery of internal standards



# PPCP QA ACCEPTANCE CRITERIA

- EPA Tier 1 Validation Protocols for Single Laboratory Method for IPR, MS/MSD, and MDL criteria
- Duplicate Criteria – 40% RPD for Analytes 10X above MDL
- Bracketing Cal. – 40% RSD for targets detected
- Cal. Ver – 25% RSD for native analytes with exact labeled standards, 35% for others
- Linearity (ICAL) – 25% RSD for native analytes that have exact labeled standards, 35% for others

# **PPCP Analyte List**

## **ESI – LC/MS/MS**

### **Acidic Extraction / + ve Ionization**

<b>ACETOMINOPHEN</b>	<b>CLAXACILLIN</b>
<b>AMPICILLIN</b>	<b>CODEINE</b>
<b>AZITHROMYCIN</b>	<b>COTININE</b>
<b>CAFFEINE</b>	<b>DEHYDRONIFEDIPINE</b>
<b>CARBADOX</b>	<b>DEPHENIHYRDAMINE</b>
<b>CARBAMAZEPINE</b>	<b>DILTIAZEM</b>
<b>CEFOTAXINE</b>	<b>DIGOXIN</b>
<b>CIPROFLAXIN</b>	<b>DIGOXIGENIN</b>

# PPCP Analyte List

## ESI – LC/MS/MS

### Acidic Extraction / + ve Ionization (cont.)

<b>ENROFLAXIN</b>	<b>NORGESTIMATE</b>
<b>ERYTHROMYCIN – H2O</b>	<b>OFLOXACIN</b>
<b>FLUMEQUINE</b>	<b>ORMETOPRIM</b>
<b>FLUOXETINE</b>	<b>OXACILLIN</b>
<b>LINCOMYCIN</b>	<b>OXOLINIC ACID</b>
<b>LOMOFLOXACIN</b>	<b>PENICILLIN G</b>
<b>MICONAZOLE</b>	<b>PENICILLIN V</b>
<b>NORFLOXACIN</b>	<b>ROXITHROMYCIN</b>

# PPCP Analyte List

## ESI – LC/MS/MS

### Acidic Extraction / + ve Ionization (cont.)

<b>SARFLOXACIN</b>	<b>TRIMETHOPRIM</b>
<b>SULFACHLOROPYRIDAZINE</b>	<b>TYLOSIN</b>
<b>SULFADIAZINE</b>	<b>VIRGINIAMYCIN</b>
<b>SULFADIMETHOXINE</b>	<b>1,7 DIMETHYLYXANTHINE</b>
<b>SULFAMERIZINE</b>	
<b>SULFAMETHAZINE</b>	
<b>SULFAMETHAZOLE</b>	
<b>THIABENDAZOLE</b>	

# PPCP Analyte List

## “Tetracyclines” by ESI - LC/MS/MS

### Acidic Extraction / - ve Ionization

<b>Chlortetracycline</b>	<b>Doxycycline</b>
<b>4–Epichlorotetracycline</b>	<b>Oxytetracycline</b>
<b>Anhydrochlortetracycline</b>	<b>4 – Epitetracycline</b>
<b>4-Epianhydrochlor-tetracycline</b>	<b>4 - Epianhydrotetracycline</b>
<b>Isochlortetracycline</b>	<b>Anydrotetracycline</b>
<b>Democlocycline</b>	<b>Mincycline</b>

# PPCP Analytes by ESI – LC/MS/MS

<b>ACIDIC EXTRACTION / -VE IONIZATION MODE</b>	<b>BASIC EXTRACTION / + VE IONIZATION MODE</b>
<b>WARFARIN</b>	<b>ALBUTEROL</b>
<b>IBUPROFEN</b>	<b>CIMETIDINE</b>
<b>GEMFIBROZIL</b>	<b>METFORMIN</b>
<b>NAPROXEN</b>	<b>RANITIDINE</b>
<b>TRICLOSAN</b>	
<b>TRICLOCARBAN</b>	

# OVERVIEW of HORMONES/STEROLS ANALYSIS in POTW BIOSOLIDS

SAMPLE SIZE = 1 GRAM WET  
(after discarding “debris”)

Surrogate Addition, dry with Sodium Sulfate  
Soxhlet 18 hrs. 40:60 Hexane:Acetone  
DCM rinses added to extract, reduce

Solvent Exchange to 5% Toluene in Hexane  
Clean-Up – Layered Alumina / Florisil Column - Concentrate  
Derivatize with 99:1 BSTFA;TMCS Reagent to produce Trimethylsilyl Ethers

ANALYSIS by HRGC / HRMS  
or GC/MS

# HORMONES / STEROLS QA ACCEPTANCE CRITERIA

- EPA Tier 1 Validation Protocols for Single Laboratory Method for IPR, MS/MSD, and MDL criteria
- Duplicate Criteria – 40% RPD for Analytes 10X above MDL
- Bracketing Cal. – 40% RSD for targets detected
- Cal. Ver – 25% RSD for native analytes with exact labeled standards, 35% for others
- Linearity (ICAL) – 25% RSD for native analytes that have exact labeled standards, 35% for others



# Hormone Targets (\*= dx surrogate)

ANDROSTENIDIENE	ESTRIOL
ANDROSTERONE	ESTRONE
COPROSTANOL	17 $\alpha$ ETHINYL ESTRADIOL *
DESOGESTRAL	MESTRANOL *
17 $\alpha$ DIHYDROEQUILIN	NORETHINDIONE *
EQUILENIN	NORGESTREL *
EQUILIN	PROGESTERONE *
17 $\alpha$ ESTRADIOL	TESTOSTERONE
17 $\beta$ ESTRADIOL *	

## **Sterol Targets (\*= dx surrogate)**

<b>CAMPESTEROL</b>	<b><math>\beta</math> – SITOSTEROL</b>
<b>CHOLESTEROL *</b>	<b><math>\beta</math> - STIGMASTRANOL</b>
<b>DESMOSTEROL</b>	<b>STIGMASTEROL</b>
<b>EPICOPROSTANOL</b>	
<b>ERGOSTEROL</b>	
<b><math>\beta</math> – ESTRADIOL – 3 - BENZOATE</b>	

# BIOSOLID DETECTION LIMITS

- PPCP in validation matrix (Peat Moss)
  - Acidic Extraction / + ve Ionization (0.1 to 50 ng/g)
  - Acidic Extraction / - ve ionization (1 to 98 ng/g)
  - Tetracyclines (2-23 ng/g)
  - Basic extraction / + ve ionization (1-56 ng/g)
  - Matrix may raise DLs 10X in biosolids, target specific
- Hormones and Sterols (10 – 2000 pg/g)
  - Hormones low, sterols high
  - LR GC/MS 5-50X higher



# PPCPs - CLEAN MATRICES vs. POTW MATRICES

- ❑ Removal of Matrix Interference is Limiting DL and Method QC factor , not instruments
- ❑ Some Options in Removal of Matrix Interference
  - ❑ Limit sample size
  - ❑ Wet weight preferred as POTW Biosolids Solids up to 95% H<sub>2</sub>O with sig. matrix
  - ❑ SPE cartridge must capture targets and surrogates, excess matrix will compete
  - ❑ Increase run times, less polar mobile phases
- ❑ Representative Matrices for Validation not Available
  - ❑ Forces clean matrices DL, IPR validation (peat, sand)
  - ❑ Experience from Method QC required

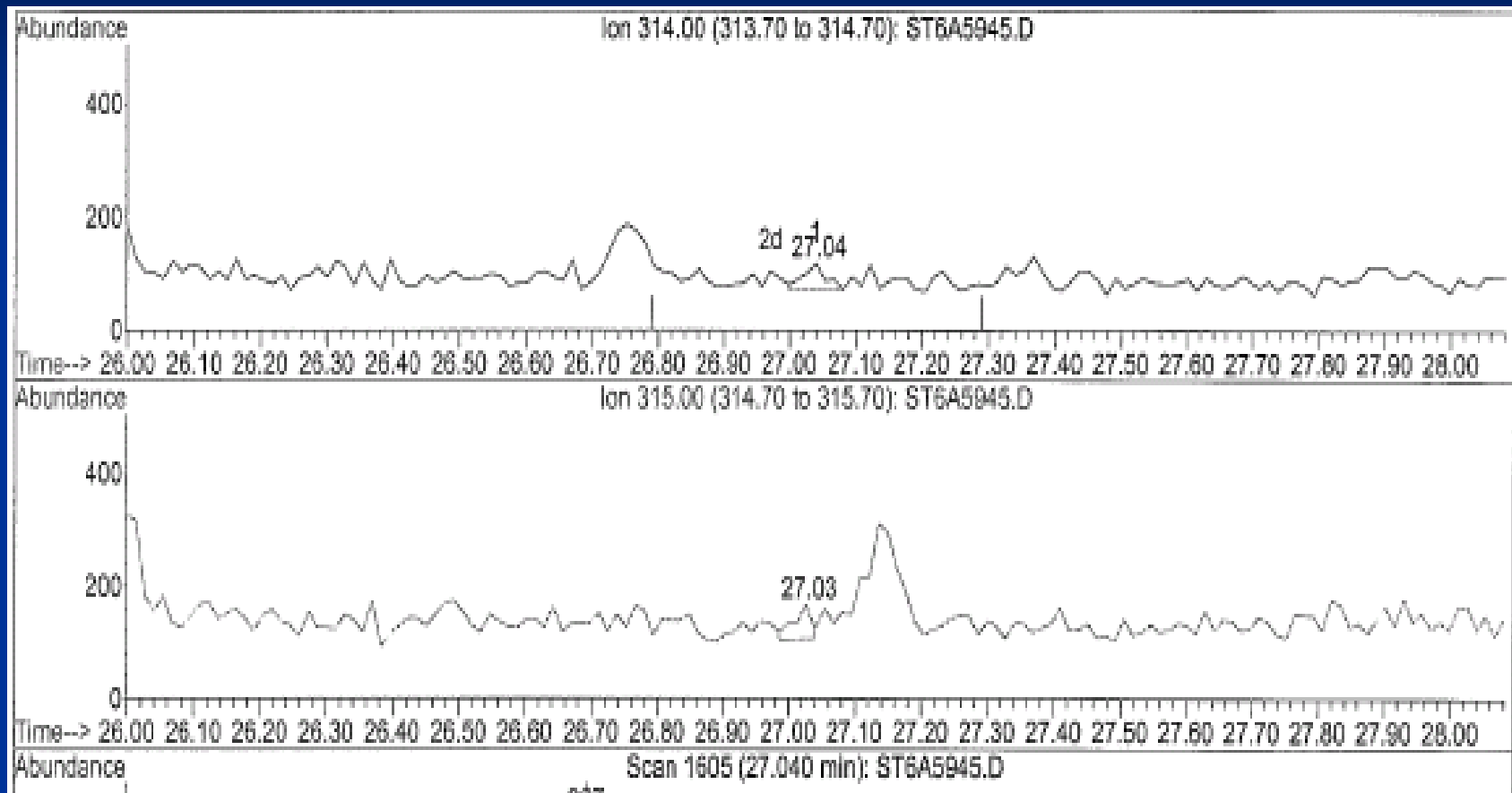


# **HORMONES and STEROLS - CLEAN MATRICES vs. POTW MATRICES**

- ❑ **Removal of matrix interference is limiting DL and Method QC factor**
  
- ❑ **Some Options in Removal of Matrix Interference**
  - ❑ **HRMS option to improve selectivity**
  - ❑ **Can be accomplished with LC/MS/MS if high level sterols not targeted with low level hormones**
  - ❑ **Water removal vital**
  - ❑ **Limited clean-up options for large target list**
  
- ❑ **Representative Matrices for Validation not Available**
  - ❑ **Clean Matrices used for DL, IPR validation (peat, sand)**
  - ❑ **Experience from method QC required**



# 17 $\beta$ Estradiol in Biosolids – GC/MS



- Native 17  $\beta$  Estradiol – “NQ”

# PPCPs – Suppression and Enhancement

- ❑ **Key effect in PPCP analysis by LC/MS/MS**
  - ❑ Wide variety in POTW biosolid chemistry / process
  - ❑ Varies widely by target
- ❑ **Controls**
  - ❑ Limit sample size
  - ❑ Increase capacity of clean-up (SPE type and size)
- ❑ **Monitoring and Correcting**
  - ❑ Recovery Standards to Monitor Surrogate Response
  - ❑ Dilution and re-injection when suppression or enhancement suspected



# PPCPs – SUPPRESSION and ENHANCEMENT

	Biosolid A		Biosolid B		Biosolid C	
Sample size	1.14 g	3.09 g	1.16 g	3.07 g	1.11 g	3.12 g
	ng/g	ng/g	ng/g	ng/g	ng/g	ng/g
Albuterol	0.2	0.1	0.2		0.4	
Cimetidine	11.9	10.3	34.1	29.6	0.2	0.3
Metformin		10.1	40.4	21.3	28.0	15.9
Ranitidine	1.2	1.1	3.6	3.0		
d3-Albuterol	95.6	78.3	85.9	67.5	119.0	125.6
d3-Cotinine	64.3	61.9	85.5	74.2	55.5	33.3
d6-Metformin	136.9	169.5	108.8	110.7	251.8	349.4



# HORMONES and STEROLS

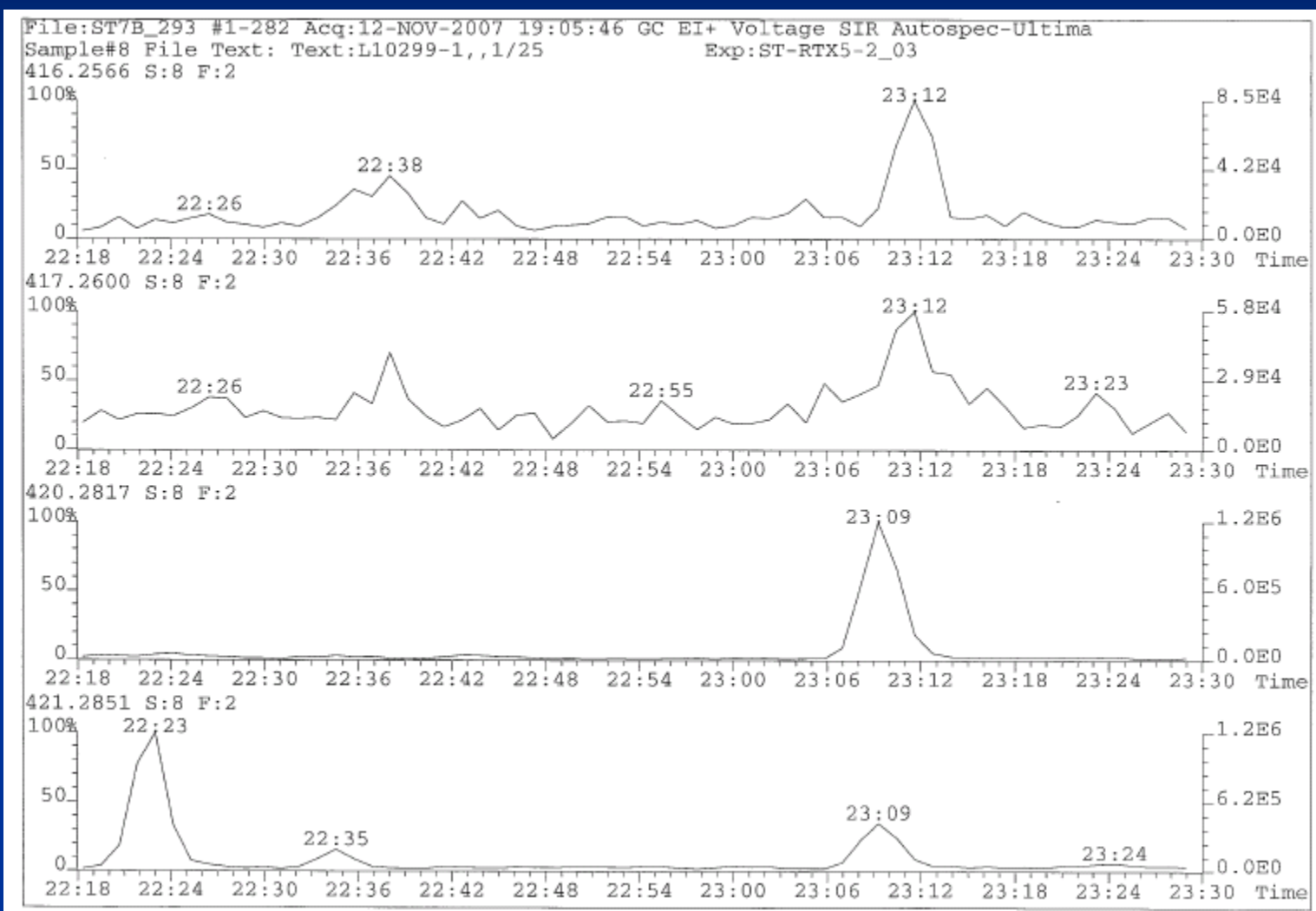
## Avoiding False Positives / Negatives

- ❑ Use of internal labeled standards and relative recovery correction
- ❑ Blank acceptance criteria from Method QC (3X standard deviation, no blank correction)
- ❑ Monitor completeness of derivatization process natives and TMS products
- ❑ Increase resolution to reduce matrix effects
  - ❑ RP of GC/MS in method 100-400
  - ❑ RP of HRMS 5000 min.



# 17 $\beta$ ESTRADIOL by HRMS

## Primary / Confirming Channels



# PPCPs – Concentration Range of Analytes

- ❑ High Levels produce need for dilution
- ❑ Create difficulties in MS/MSD for high level analytes
- ❑ Frequent detects above 1000 ng/g in Biosolids
  - ❑ Ciproflaxin, Ofloxacin, Triclocarban, Triclosin, ETC, Doxycycline
- ❑ Frequent detects above 1000 ng/L in POTW influent
  - ❑ Acetaminophen, Caffeine, Ibuprofen, Cotinine, Trimethoprim, Carbamazepine, Metformin, Sulfamethoxazole



# **HORMONES and STEROLS – Concentration Range of Analytes**

- ❑ **Hormones low level, many sterols very high level in POTW biosolids**
- ❑ **Affects blank acceptance criteria**
  - ❑ **5-10 ng for most compounds**
  - ❑ **500 for cholesterol and beta sitosterol**
- ❑ **Adjustments required to prevent “NQ” on high level sterols (cholesterol and beta sitosterol most frequent)**
- ❑ **Extract split used to maintain QC**
  - ❑ **Over spike with Sterol surrogates at beginning of process**
  - ❑ **1/20 split after clean-up**
  - ❑ **2 instrument runs**
  - ❑ **Method QC adjusted to match**



# SAMPLE HANDLING / PRESERVATION

- Freeze biosolid samples immediately
  - Arrest Microbiological activity
  - No definitive studies indicating hold times
  - Glass containers (PPCP and Hormones/Sterols)
- Extract immediately on thawing
- POTW processes homogenize sample
  - Allows minimization of sample size
  - Sampling plan to determine repeatability
- Have a strategy for “free water”
  - Discard, 2 analysis, attempt to re-homogenize



# CONCLUSIONS

- Viable low level detection methods available for large panels of PPCPs and Hormones / Sterols
- Basic methods applicable for environmental levels (water, soil, and sediment) and POTW matrices (Influent, Effluent, Biosolids)
- High variability in POTW sludge matrix effects and levels, much work required to characterize vs. specific attributes
- High variability requires robust methods and “coping” strategies



## NEXT STEPS

- Current - Peer Review of method performed by EPA – Q3/4 2007
- Further processing of samples to develop further statistical information
- Detailed Information targeted for publication by AXYS clients
- Multi-Lab Validation?
- Metabolites and Degradation Products
- Questions?



# ACKNOWLEDGEMENTS

- *AXYS Laboratory staff*
  - 20 staff over 3 year period
- Those who have contributed to literature on this subject

